

Optimization of an FaeG DNA vaccine in pigs

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List of abbreviations

A	Adenine
Ab	Antibody
Act A	Actin nucleator
Ag	Antigen
APC	Antigen-presenting cell(s)
AS	Antisense
ASC	Antibody-secreting cell(s)
AttHRV	Attenuated human rotavirus
BAC	Bacterial arteficial chromosome
BFRf	Bacterioferritin
BGH	Bovine growth hormone
BHV	Bovine herpes virus
C	Cytosine
cAMP	Cyclic AMP
CD	Cluster of differentiation
Cerv LN	Cervical superficial dorsal lymph node
CMI	Cell-mediated immunity
CMV	Cytomegalovirus
CMV IE	Cytomegalovirus immediate early
ConA	Concanavalin A
Cpm	Counts per minute
CSFV	Classical swine fever virus
CT	Cholera toxin
CTL	Cytotoxic T lymphocyte(s)
DC	Dendritic cell
DNA	Deoxyribonucleic acid
Dppi	Days post primary immunization
<i>E. coli</i>	<i>Escherichia coli</i>
ELISA	Enzyme-linked immunosorbent assay
ELIspot	Enzyme-linked immunospot
ETEC	Enterotoxigenic <i>Escherichia coli</i>
FITC	Fluorescein isothiocyanate

FMDV	Foot and mouth disease virus
F4R	F4 receptor
G	guanine
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP/gp/g	Glycoprotein
HA	Hemagglutinin
HBsAg	Hepatitis B surface antigen
HGH	Human growth hormone
HP-NAP	<i>Helicobacter pylori</i> neutrophil activating protein
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HRV	Human rotavirus
HSV	Herpes simplex virus
IBDV	Infectious bursal disease virus
ID	Intradermal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IPP	Ileal Peyer's patch
JPP	Jejunal Peyer's patches
LC	Langerhans' cell
LLO	Listeriolysin
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>
LN	Lymph node
LP	Lamina propria
LT	Heat-labile enterotoxin
LT vectors	Plasmids encoding the A and B subunits of LT
MAb	Monoclonal antibody
MC	Monomorphonuclear cells
MCMV	Murine cytomegalovirus
MCS	Multi cloning site
Mes LN	Mesenteric lymph node

MHC	Major histocompatibility complex
MHCI	Major histocompatibility complex class I
MHCII	Major histocompatibility complex class II
MOMP	Major outer membrane protein
MPB	Mycobacterial protein secreted by <i>Mycobacterium bovis</i>
MPT	Mycobacterial protein secreted by <i>Mycobacterium tuberculosis</i>
NA	Not applicable
ND	Not determined
NF	Normalization factor
NK cell	Natural killer cell
NP	Nucleoprotein
NrdF	Ribonucleotide reductase R2 subunit
NS	Non-structural
ODN	Oligodeoxynucleotide
ORFV	Orf virus, parapoxvirus
OVA	Ovalbumin
P97	Adhesin repeat region R1
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
Pc	Post-challenge
PCV2	Porcine circovirus type 2
Pi	Post-immunization
PLG	Poly(lactide-co-glycolide)
PolyA	Polyadenylation site
PP	Peyer's patch
ProT	Prothymosine α
PRRSV	Porcine reproductive and respiratory syndrome virus
PRV	Pseudorabies virus
Ppi	Post-primary immunization
Psi	Post-secondary immunization
PWD	Postweaning diarrhoea
RPAV	Recombinant porcine adenovirus
Rvac	Recombinant vaccinia virus
S	Sense

S1P	Sphingosine-1-phosphate
Sc	Subcutaneous
<i>S. cholerasuis</i>	<i>Salmonella cholerasuis</i>
SEM	Standard error of the mean
SD	Standard deviation
<i>S. flexneri</i>	<i>Shigella flexneri</i>
SFV	Semliki forest virus
sIgA	Secretory immunoglobulin A
SI	Stimulation index(es)
SIN	Sindbis virus
ST	Heat-stabile enterotoxin
<i>S. typhi</i>	<i>Salmonella typhi</i>
<i>S. typhimurium</i>	<i>Salmonella typhimurium</i>
SWC	Swine workshop cluster
T	Thymine
TCR	T cell receptor
TGF	Tumor growth factor
Th	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
tPA	Human tissue plasminogen activator
UEA-1	Ulex europaeus agglutinin 1
VitD3	1 α ,25-dihydroxyvitamin D ₃ , calcitriol
VN	Virus neutralizing antibodies
VP	Viral protein
<i>Y. enterocolitica</i>	<i>Yersinia enterocolitica</i>

PART I

INTRODUCTION

Introduction

Intestinal infections with enterotoxigenic *Escherichia coli* (ETEC) affect neonatal and recently weaned piglets. These infections cause diarrhoea and are responsible for severe economic loss due to growth retardation, elevated drug use and mortality. In general, most neonatal infections can be prevented by passive colostral and lactogenic immunity obtained by vaccination of the sow. However, this passive protection decreases with aging and at weaning lactogenic immunity disappears. Consequently, the newly weaned piglet becomes highly susceptible to enteropathogens. In Belgium, the losses due to ETEC induced postweaning diarrhoea (PWD) are estimated to be approximately 13 000 000 euro/year (Snoeck, 2004).

PWD occurs within 10 days of weaning, often the first 4-5 days after weaning and is characterised by greyish brownish or watery diarrhoea (Hampson, 1994). The diarrhoea is usually transient and resolves within 3-5 days but may persist and cause death from dehydration. Even when the diarrhoea is transient, marked reduction in the rate of daily weight gain may occur. ETEC are the most important pathogens in the PWD complex. They can be carried into the weaner house by healthy suckling pigs which have the organism in their lower part of the intestinal tract (Hampson *et al.*, 1985). Upon weaning, proliferation of these ETEC can occur with spread of the bacteria to other pigs. However, colonisation of the recently weaned pigs can also occur following their exposure to the contaminated environment of a weaner house that has contained infected pigs (Hampson *et al.*, 1987) or by mechanical transmission of the bacteria.

Porcine pathogenic *E. coli* involved in PWD typically belong to serogroups O8, O138, O139, O141, O147, O149 and O157, of which O149 seems to be the predominant serogroup in most countries (Sojka, 1965; Blanco *et al.*, 1997; Frydendahl *et al.*, 2002; Naomani *et al.*, 2003). ETEC can cause severe diarrhoea in newborn and weaned piglets by the production of heat-labile enterotoxin (LT) and/or heat-stable enterotoxins (STa or STb). These enterotoxins are extracellular proteins or peptides, which are able to cause diarrhoea by changing the water and electrolyte balance of the small intestine (Blanco *et al.*, 1997).

The bacteria possess adhesins which enable them to bind specific receptors on the intestinal epithelium and subsequently to colonize the small intestine. Of these adhesins, F4 (K88) fimbriae are the best characterised (Van den Broeck *et al.*, 2000). The F4 fimbriae are long proteinaceous appendages mainly composed of several hundreds identical 27.5 kDa FaeG subunits. Some parts of the FaeG subunits are conserved, whereas in other regions a

certain degree of variation exists. As a consequence, 3 antigenic variants of F4 have been identified, called F4ab, F4ac and F4ad (Ørskov *et al.*, 1964; Guinée and Jansen, 1979). Among these, F4ac is the most prevalent (Westerman *et al.*, 1988).

The receptors for F4⁺ ETEC are not present in every pig and absence of the F4-receptors (F4R) causes resistance to F4⁺ ETEC-induced diarrhoea. Presence of the F4R gene is genetically determined, with presence of this gene being the dominant character (Gibbons *et al.*, 1977). However, expression of the F4R gene is influenced by epistatic genes, causing variation in the degree of F4R expression (Bijlsma and Bouw, 1987). Pigs become resistant to F4⁺ ETEC infection with increasing age by release of higher amounts of F4R in the intestinal mucus layers (Conway *et al.*, 1990).

Although ETEC play a central role in PWD, the etiology of PWD is more complex as several weaning-associated factors predispose the pig to PWD. (1) The withdrawal of specific (anti-adhesin and anti-toxin antibodies) and non-specific (anti-bacterial agents such as lactoferrin and transferrin) protective effects contained in sow's milk (Nagy *et al.*, 1979; Deprez *et al.*, 1986). (2) A temporarily decrease in gastric acidity, allowing ingested bacteria to survive and to gain access to the small intestine (Schulman, 1973). (3) A lack of dietary intake (Kelly *et al.*, 1984), hypersensitivity to new dietary antigens (Miller *et al.*, 1984), change from a liquid (milk) diet to a dry diet, an inflammatory response to bacterial toxins and/or digestion products (Kenworthy, 1976) or viral infection (Lecce *et al.*, 1982) resulting in changes in structure of the small intestine and enterocyte brush border enzyme activities. These changes include a decrease of the villus height, increase of the crypt depth, decrease of brush border lactose and sucrase activity, sodium-dependent alanine transport and capacity for xylose absorption. The net result is a reduction in the intestinal digestive and absorptive function encouraging the development of osmotic diarrhoea, whilst the unabsorbed intestinal content acts as substrate for ETEC (Miller *et al.*, 1986). (4) The incompletely developed large intestinal microflora causing a limited fermentation, diminishing water and electrolyte adsorption (Hampson, 1987). (5) The amount of diet consumed and the physical form of the diet. It is suggested that there is a positive correlation between the amount of diet consumed and the duration of faecal ETEC excretion and the occurrence of PWD (Hampson and Smith, 1986). On the other hand, the changes in small intestinal structure and enzyme activities are reduced if the weaner diet is fed as a liquid slurry (Deprez *et al.*, 1987). (6) Exposing the pigs to cold temperatures or temperature fluctuations (Wathes *et al.*, 1989).

It was previously shown that oral immunization of weaned F4-receptor positive (F4R⁺) pigs with F4 fimbriae results in an antigen-specific secretory IgA (sIgA) response at the intestinal mucosa, protecting these pigs against a subsequent F4⁺ ETEC challenge (Van den Broeck *et al.*, 1999a). However to protect newly weaned pigs, the immune system should already be primed during the suckling period. Most suckling pigs have maternal antibodies against F4 fimbriae, hampering their use as a vaccine.

DNA vaccines can reach their target cells in the presence of maternal antibodies and produce their encoded antigen endogenously, so maternal antibodies won't interfere with the induction of cellular immunity by these vaccines. Therefore, we suggested that priming with a DNA vaccine encoding the adhesive subunit of F4, the FaeG, could be an interesting approach to obtain protective immunity against PWD.

The best route to induce an intestinal mucosal immune response is the oral route. However, oral DNA vaccination is difficult as it involves overcoming several challenges. These include depurination by low pH in the stomach, enzymatic degradation by DNases in the gut, crossing the physical barrier imposed by the mucus layer and overcoming the epithelial barrier to reach the intestinal mucosal inductive sites. Parenteral vaccination seems more appropriate, but this stimulates the systemic rather than the mucosal immune system. However, when using protein vaccines, parenteral vaccination has successfully induced mucosal responses if a mucosal boost or immunomodulating adjuvants were included. One such adjuvant is 1 α ,25(OH)₂D₃, the active form of vitamin D₃ (vitD₃). When added to protein antigens, vitD₃ appears to modulate a systemic immune response towards a mucosal one in mice (Daynes and Araneo, 1994; Daynes *et al.*, 1996; Enioutina *et al.*, 1999, 2000) and to a lesser extent in pigs (Van der Stede *et al.*, 2001, 2003, 2004). Although vitD₃ has traditionally been associated with calcium homeostasis, it is now appreciated to exert pronounced effects on the mammalian immune system. VitD₃ exerts its actions through a nuclear vitamin D receptor (nVDR) (Bouillon *et al.*, 1995) present on most cells of the immune system such as monocytes, macrophages, dendritic cells (DC) (Adorini *et al.*, 2001), T- and B-lymphocytes (Morgan *et al.*, 1994, 1996, 1999). VitD₃ stimulates the differentiation and maturation of monocytes (Abe *et al.*, 1981) while DC differentiated from bone marrow precursors in presence of vitD₃ acquire a less mature phenotype (Berer *et al.*, 2000; Griffin *et al.*, 2000, 2003). VitD₃ also suppresses T cell proliferation as well as production of the T-helper 1 (Th1) cytokines IFN- γ and IL-2 (van Etten and Mathieu, 2005; Bhalla *et al.*, 1986; Reichel *et al.*, 1987). On the other hand, vitD₃ is able to enhance the production of Th2 cytokines such as IL-4 and IL-10 (Daynes and Araneo, 1994; Daynes *et al.*, 1996; Cantorna *et al.*, 1998; Adorini *et al.*, 2001) as

well as of the Th3 cytokine TGF- β (Fukaura *et al.*, 1996; Weinreich *et al.*, 1999). TGF- β and IL-10 are involved in IgA isotype switching (Defrance *et al.*, 1992). Consequently, vitD₃ is able to mimic in the local draining lymph node (LN) the cytokine pattern normally produced in the intestinal Peyer's patches following oral immunization, which was originally believed to promote the mucosal response to parenteral protein vaccines (Daynes and Araneo, 1994; Daynes *et al.*, 1996). Later, the same group demonstrated that mainly the migration of antigen-pulsed DC to the Peyer's patches and the activation and differentiation of antigen-specific B cells in these tissues, is responsible for the induction of the mucosal response to parenteral vaccines (Enioutina *et al.*, 1999, 2000). Antigen uptake by DC is normally followed by a maturation process that includes a decline in expression of inflammatory chemokine receptors allowing the release of DC from the immunization site, with a parallel upregulation of the chemokine receptor CCR7 and enhanced responsiveness to sphingosine-1-phosphate (S1P) resulting in their traffic through the afferent lymphatic vessels and their eventual sequestration into a draining LN (Caux *et al.*, 2002; Czeloth *et al.*, 2005). However when DC are stimulated in tissue environments containing vitD₃, they are capable of avoiding draining LN sequestration. Mechanisms responsible for this altered migratory pattern include a reduced expression level of CCR7, a temporary inhibition of chemotaxis towards CCR7 ligands but not towards S1P and an upregulated surface expression of the $\alpha_4\beta_7$ integrin responsible for homing to mucosa-associated lymphoid tissues (Enioutina *et al.*, 2007). The ability to change the migratory properties of antigen-pulsed DC is not unique to vitD₃, but also seems to occur when enhancers of cyclic AMP (cAMP) such as cholera toxin, LT or forskolin are added to cutaneously administered protein vaccines (Enioutina *et al.*, 2000; Belyakov *et al.*, 2004). Enioutina *et al.* (2000) hypothesized that this might be due to the capacity of cAMP to stimulate the expression of 1, α -hydroxylase, which converts the normal circulating 25(OH)D₃ into vitD₃. However, such a relationship between the ability of vitD₃ and cAMP to promote priming of mucosal responses remains to be determined.

In the present thesis, we examined if parenteral FaeG DNA vaccination in the presence or absence of immunomodulating adjuvants could prime an intestinal mucosal immune response. The pcDNA1/*faeG19* DNA vaccine previously developed at our lab only induced marginal immune responses following parenteral immunization of pigs. In order to enlarge our chances on successful mucosal priming, we first applied several strategies to enhance the immunogenicity of our FaeG DNA vaccine. Furthermore, given the critical role of antigen-presenting cells (APC) in the induction of mucosal immunity, granulocyte-macrophage colony-stimulating factor, a well known chemo-attractant of APC was included in our

vaccination strategy. Part I (Chapter 1) reviews the literature on DNA vaccination. In part II, the specific aims of the study are described. The experimental work is presented in part III (chapter 2 to 5). Part IV contains the general discussion and the overall conclusions and future perspectives (chapter 6).

CHAPTER 1

DNA vaccination in the pig

1.1 Introduction

The concept of DNA as a vaccine originates from the observation that delivery of DNA into an animal could lead to *in vivo* gene expression. In the 1950's, experiments aiming to understand the fundamental nature of cancer tested the delivery of crude tumour DNA preparations to rodents and observed tumour growth (Stasney *et al.*, 1950; Ito *et al.*, 1961). A decade later, Atanasiu and colleagues extended these early observations by the finding that subcutaneous administration of crude polyoma virus DNA into hamsters not only resulted in tumour development but also in the generation of antibodies against the virus (Atanasiu *et al.*, 1962; Orth *et al.*, 1964). Later Israel *et al.* (1979) extended the initial findings to recombinant viral DNA. All together, these data suggested the idea of DNA transfer to drive protein expression and activate the immune system.

In the early 1980's, two independent studies demonstrated the *in vivo* expression and the biological activity of plasmid encoded gene products (Will *et al.*, 1982; Nicolau *et al.*, 1983). However, the major turning point regarding the *in vivo* expression of plasmid encoded genes comes from a study by Wolff *et al.* (1990) reporting that the *in vivo* activity of a reporter gene could be detected for up to 2 months following intramuscular 'plasmid or naked' DNA injection into mice. Their data suggested that, at least in mice, antigens could be delivered at high enough levels to give persistent signals.

In 1992, a critical study published by Tang *et al.* (1992) utilizing DNA coated gold beads to transfect cells *in vivo* reported the generation of an antibody response against the DNA encoded proteins. This manuscript highlighted that small amounts of DNA delivered to the skin could stimulate an antigen specific humoral immune response. Soon thereafter, several investigators demonstrated the induction of both humoral and cellular responses against true human pathogens like influenza (Ulmer *et al.*, 1993; Fynan *et al.*, 1993), HIV (Wang *et al.*, 1993) and hepatitis B (Davis *et al.*, 1994). Importantly, these studies also showed that DNA vaccination could impact on viral replication. Since then, DNA vaccines have been reported to induce protective immunity against an array of pathogens, including viruses, parasites and bacteria (Donnelly *et al.*, 1997; Watts and Kennedy, 1999). However, most of these studies were performed in mice, whereas experiments in large animals and man were generally less successful, especially with regard to antibody responses. Therefore, improving the immune potency and deliverability of DNA vaccines has become a central issue during the last decade and new formulations, new adjuvants and more sophisticated constructs have been developed (Laddy and Weiner, 2006; Liu *et al.*, 2006; Leitner *et al.*, 1999). However, studies have demonstrated that the success of DNA vaccines to induce immune responses in mice still does

not necessarily translate itself to large animals and man (Laddy and Weiner, 2006). Nevertheless, two DNA vaccines have recently been approved for veterinary use; one for West Nile Virus in horses (Powell, 2004) and a second for Infectious Haematopoietic Necrosis in salmon (Lorenzen and LaPatra 2005). Hence, DNA vaccines finally appear to gain a position on the market. However, the challenge remains to make future vaccines more potent and cheaper to manufacture to allow them to become routinely used.

1.2 Principle of DNA vaccines

The principle of DNA vaccination is relatively simple: a gene encoding a specific antigen is inserted into a DNA plasmid that can be replicated in bacteria, purified and directly inoculated into the host to be vaccinated. The plasmid insert then becomes transcribed and translated by the host cells and the protein produced initiates an immune response. However, many details regarding the uptake of the plasmid into the host cell and the following transport to the nucleus remain unclear.

Plasmid design

The DNA plasmids are bacteria-derived, circular DNA molecules equipped with a unit for plasmid propagation in bacteria and a transcriptional unit to allow eukaryotic expression (Figure 1). The transcriptional unit minimally includes a strong eukaryotic or viral promoter to drive transcription in mammalian cells, a multiple cloning site to allow the insertion of foreign genes and a polyadenylation sequence to provide stabilization of mRNA transcripts. These sequences are discussed in more detail later (1.5.1, vector elements). The unit for plasmid propagation in bacteria includes an origin of replication and a marker to enable plasmid selection during bacterial growth. Often, the *E. coli* ColE1 origin of replication is used because it provides high copy numbers, allowing the purification of high yields of plasmid DNA. As selection markers, bacterial antibiotic resistance genes are most commonly used. Since it is recommended to avoid resistance to therapeutic antibiotics, kanamycin or neomycin resistance genes are better suited for this purpose than the frequently used ampicillin resistance gene.

In addition, bacterial plasmid DNA contains immunostimulatory CpG motifs which may enhance the immunogenicity of the DNA vaccine (Sato *et al.*, 1996).

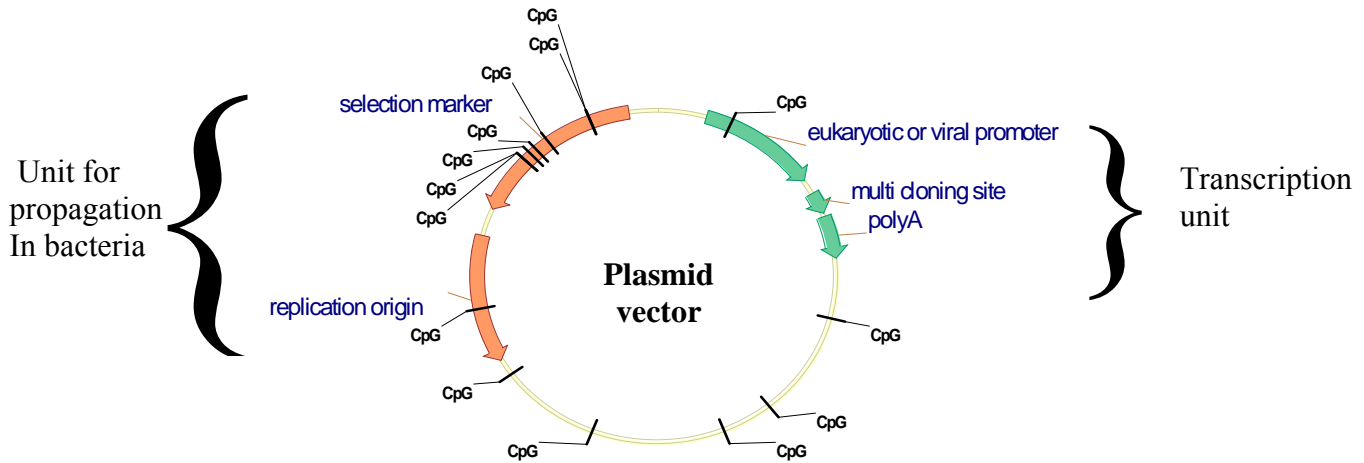


Figure 1: Schematic representation of the basic requirements of a plasmid DNA vector. Essential features include a transcriptional unit, consisting of a strong promoter active in eukaryotic cells, a multi cloning site, a polyadenylation sequence (polyA), and a unit for plasmid propagation in bacteria, consisting of an origin of replication in bacterial cells and a selection marker. The presence of CpG motifs in the plasmid backbone provides adjuvant properties to the DNA vaccine.

The role of CpG motifs

In bacterial DNA, CpG dinucleotides are unmethylated and present at a high frequency (1/16 bases), whereas they are under-represented in mammalian DNA. These differences in combination with specific flanking bases constitute a CpG motif that is recognized as a 'danger' signal by the innate immune system. The basic formula for such a CpG motif has been described as 5'-X₁X₂**CG**Y₁Y₂-3', where X₁ is a purine (adenine (A) or guanine (G)), X₂ is a purine or a thymine (T), while Y₁ and Y₂ are pyrimidines (cytosine (C) or T) (Krieg *et al.*, 2000). The recognition of CpG-motifs by the innate immune system is species-specific (Kanellos *et al.*, 1999; Bauer *et al.*, 2001, Klinman *et al.*, 2004) and CpG motifs immunomodulatory for different animal species are summarized by Cox *et al.* (2006). Activation of the innate immune system by these motifs requires engagement of the Toll-like receptor 9 (TLR-9), which activates intracellular signalling pathways resulting in the induction of several transcriptional activators (Hemmi *et al.*, 2000).

The multiple effects of CpG motifs on cells of the immune system are summarized in Figure 2. Briefly, CpG oligodeoxynucleotide (ODN) act directly on a small subset of cells, including B lymphocytes, monocytes, macrophages, DC, natural killer (NK) cells and mast cells. CpG motifs stimulate B cells to proliferate and secrete IgG, interleukin (IL)-6 and IL-10 (Klinman *et al.*, 1996). They also directly activate monocyte/macrophages and dendritic cells

to secrete interferon (IFN)- α , IL-6, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokines and tumor necrosis factor (TNF)- α . Similarly, either directly or through the cytokines produced by APC, they stimulate NK cells to produce IFN- γ and to increase killer activity. In addition to their ability to support cytokine and Ig secretion, CpG motifs could contribute to the development of an immune response by upregulating cell surface expression of MHC class II (MHCII) and other co-stimulatory molecules, such as B7.1 and B7.2. As a result of these activities, CpG motifs rapidly stimulate host defenses and polarize immune responses to a T-helper (Th)1-like response in both DNA vaccines as well as if used as an adjuvant with protein vaccines (Weiner *et al.*, 1997; Krieg *et al.*, 1998).

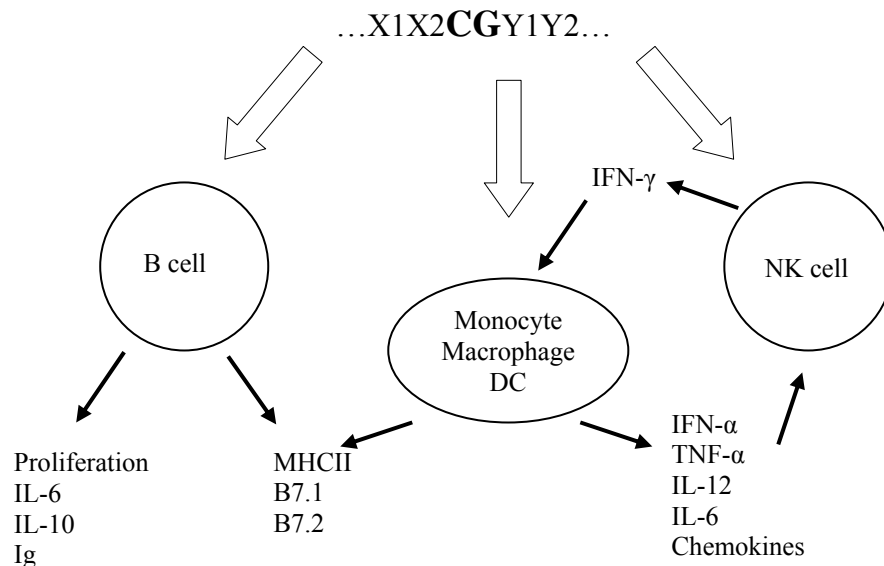


Figure 2. Effects of CpG motifs on leukocytes. CpG motifs stimulate B cells to proliferate and secrete IgG, IL-6 and IL-10. They directly activate monocytenomacrophages and dendritic cells to secrete IFN- α , IL-6, IL-12, GM-CSF, chemokines and TNF- α . Either directly or through the cytokines produced by APC, they stimulate NK cells to produce IFN- γ and to increase killer activity. In addition, CpG motifs induce upregulation of cell surface expression of MHCII and other co-stimulatory molecules, such as B7.1 and B7.2.

1.3 Immunogenicity of DNA vaccines

The ability of DNA vaccines to elicit antibody responses and both CD4⁺ and CD8⁺ T cell responses has been well documented. Each of these responses generally require the antigen to be encountered differently: B cells can directly recognize the antigen, whereas CD4⁺ T cells

primarily recognize peptide-MHC class II complexes on the surface of antigen-presenting cells (APC) that have taken up and processed exogenous antigens, and CD8⁺ T cells are generally restricted to peptide-MHC class I complexes derived from endogenous proteins that have undergone proteasome-dependent intracellular processing. The exact mechanism by which DNA vaccination results in the activation of these 3 arms of the immune system remains to be determined.

Role for somatic cells in the induction of immunity following DNA vaccination

Somatic cells (for example keratinocytes, myocytes or other MHC class II-negative cells) are the primary cell type that becomes transfected following DNA vaccination. Although these cells express MHC class I molecules, they generally don't express costimulatory molecules and thus are unlikely to function as effective APC (Pardoll and Beckerleg, 1995). Evidence for this was first provided by studies using bone marrow chimeras. In a study by Corr and colleagues (1996), parent into F1 bone marrow chimeras were generated by destroying the bone marrow of H-2^{bxd} F1 mice by irradiation and substituting them with either parental H-2^b or parental H-2^d T cell-depleted bone marrow. So, these mice had myocytes whose MHC molecules expressed both parental haplotypes, T cells that recognize both parental haplotypes (due to an F1 thymus) and APC expressing MHC molecules from only one of the parents. The CTL response generated on subsequent DNA vaccination of these mice was found to be restricted to the haplotype of the reconstituted bone marrow. Similar findings were observed for epidermal gene gun immunization (Iwasaki *et al.*, 1997). These data strongly indicate that myocytes and keratinocytes are not converted into APC by DNA transfection and that bone marrow-derived APC are necessary to prime CTL responses after genetic immunization.

In studies attempting to more clearly define the exact role of myocytes or keratinocytes in the induction of an immune response following DNA vaccination, Torres *et al* (1997) reported that needle-injected muscle tissue is not critical to raise responses, whereas gene gun-bombarded skin is. Indeed, excision of the injected muscle within 10 minutes of DNA inoculation did not affect cellular or humoral responses. This implies that the events which lead to immune responses following intramuscular DNA vaccination take place in more distal tissues and are either initiated by rapidly migrating free DNA, by transfected hemopoietic cells in blood or by transfected APC migrating from the injection site into blood or lymph (Torres *et al.*, 1997). Although the injected muscle is known to retain plasmid DNA and to produce antigen for up to 18 months (Wolff *et al.*, 1990), the data by Torres *et al* suggested that the muscular antigen production is not needed to induce immune responses and

consequently that the antigen producing cells responsible for immune induction remain to be identified.

On the contrary, they demonstrated that the integrity of the targetted skin was required for at least 3 days to induce maximal responses following gene gun immunization. This correlates with the temporal expression of the antigen in the skin and could reflect the time keratinocytes serve as antigen factories or the time it takes directly transfected Langerhans' cells (LC) to migrate out of skin, or both (Torres *et al.*, 1997). At least some role for directly transfected LC is suggested by the appearance of DNA-expressing DC in draining lymph nodes within 24 h of gene gun bombardment (Condon *et al.*, 1996), whereas a role for keratinocytes as antigen factories was proven by the use of a keratinocyte-specific promoter (K14). Antigen expression driven by this promoter generated comparable humoral and cellular responses than antigen expression driven by the frequently used cytomegalovirus (CMV) promoter, which is active in all eukaryotic cell types (Hon *et al.*, 2005). In addition, the data by Hon and colleagues demonstrated that also B cells play a strong role in cross-priming T cell responses from keratinocyte-derived antigen. Indeed, in addition to DCs, the B cell population isolated from the draining LN of mice immunized with both the CMV and the keratinocyte-specific expression construct induced significant CD8⁺ T cell proliferation. Furthermore, B cell-deficient mice immunized with the keratinocyte-specific construct showed only minimal CD4⁺ and CD8⁺ T cell responses.

The importance of skin but not muscle to DNA-raised responses might reflect the better immune surveillance function of the skin. Indeed, LC constitute about 5% of the epidermal cells. Furthermore, keratinocytes are specialized to enhance the immune surveillance function by secreting cytokines in response to injury or infection. These cytokines upregulate MHC class II molecules on LC and stimulate LC migration (Salmon *et al.*, 1994; Belsito *et al.*, 1989). In muscle, the lower frequency of resident DC or the low ability of transfected myocytes to enhance the recruitment and activation of APC may account for the minimal role of muscle in DNA vaccination. However, an important limitation of all these studies is that they were performed in mice in which the intramuscular immunization generally includes the injection of a large volume (typically 50-100 µl) into a relatively small muscle. This makes it likely that DNA becomes forced out of the muscle into the vessels and other surrounding tissues. Indeed, one hour post-vaccination, plasmid DNA could already be detected in most highly vasculated tissues, including blood, bone marrow and spleen (Parker *et al.*, 1999). Furthermore, it should be mentioned that when surveying muscle-associated plasmid, these

studies only focused on the injected muscle, whereas muscle tissue separate from the injection site might also be transfected.

Professional APC are responsible for priming cellular and humoral responses following DNA vaccination

There are at least 3 different ways by which APC might be involved in T cell priming: they can capture antigen secreted by other transfected cells, resulting in MHC class II-restricted presentation, pick up antigen from transfected cells by an as yet ill-defined mechanism, resulting in MHC class I-restricted “cross”-presentation or they can be transfected themselves, resulting in MHC class I presentation of plasmid encoded antigens (Figure 3) (Takashima and Morita, 1999).

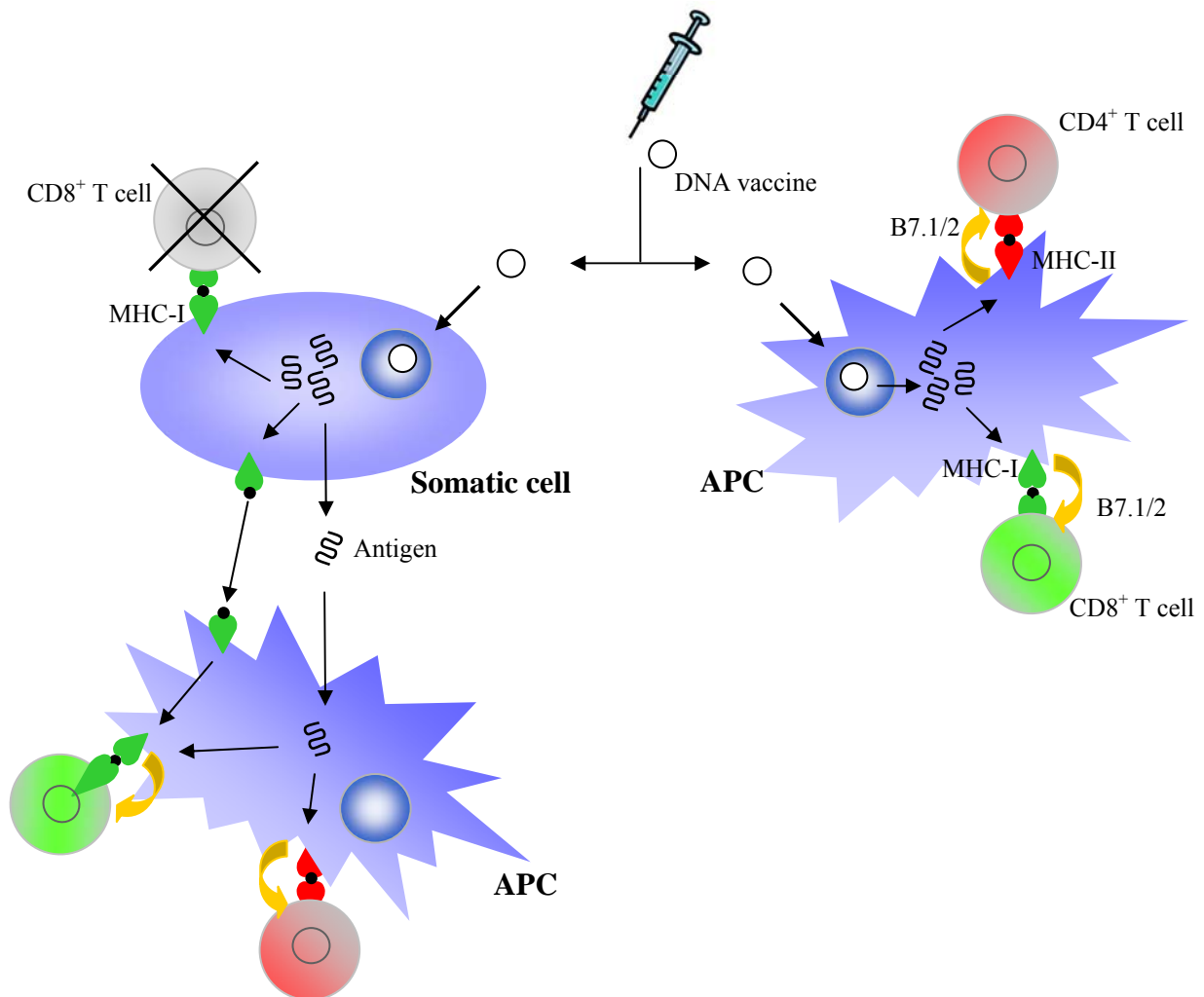


Figure 3. CD4⁺ and CD8⁺ T cell activation by DNA vaccination. Following transfection of somatic cells (left), antigen can be processed and presented to CD8⁺ T cells via MHC-I molecules. However, as these cells do not express B7.1 and/or B7.2 costimulatory molecules, they are not able to activate naive CD8⁺ T cells. APC express costimulatory molecules and can activate CD8⁺ as well as CD4⁺ T cells in different ways. They can capture antigen secreted by other transfected cells resulting in MHC class II-restricted presentation, pick up antigen from transfected cells in an as yet ill-defined mechanism resulting in MHC class I-restricted cross-presentation or they can become transfected themselves, resulting in MHC class I presentation of plasmid encoded antigen. More detailed information can be found in the text.

Several studies undertaken to clarify the contribution of direct priming and “cross”-priming in the induction of CD8⁺ T cell responses support both mechanisms. Evidence for direct priming was provided in a study by Bot *et al* (2000). In that study, mice were intradermally injected with plasmid DNA and migratory cells were subsequently analyzed for coexpression of the plasmid encoded antigen and MHC class II molecules. Upon adoptive transfer into naïve recipient mice, transfected MHC class II⁺ cells were far more efficient at priming cytotoxic T lymphocyte (CTL) responses as compared to MHC class II⁻ cells, arguing in favour of direct priming. Direct priming was also suggested by earlier studies demonstrating the migration of *in situ* transfected (DNA containing) DC to the local draining lymph nodes (Condon *et al.*, 1996; Casares *et al.*, 1997; Akbari *et al.*, 1999). Such cells were able to activate specific CD8⁺ T cells (Casares *et al.*, 1997; Akbari *et al.*, 1999; Chattergoon *et al.*, 1998). The role of directly transfected DC in the induction of CTL responses was further pinpointed by co-transfection with plasmid encoding an antigen and plasmid DNA encoding a membrane marker allowing for the selective depletion of directly transfected cells (Porgador *et al.*, 1998). In that study depletion of transfected cells from total or DC enriched LN cells reduced presentation by 60-70%.

In contrast, other studies point to cross presentation as the main mechanism by which DNA vaccines prime immune responses. To evaluate whether direct transfection of migratory cells was necessary to prime CTL responses, Corr *et al* (1999) used an expression-suppressible construct that allowed to separate transport of the plasmid versus this of the protein from the injection site. Mice lacking B and T cells were immunized in the presence or absence of the suppressor. Splenocytes (APC) from these mice were injected into recipient mice and assessed for their ability to prime CTL responses. Recipient mice that received splenocytes from mice without suppressor mounted a greater CTL response, suggesting that exogenous transfer rather than endogenous production was important for the magnitude of the response. Similarly, studies comprising transplantation of transfected myoblasts (Ulmer *et al.*, 1996; 1997) and tumor cells (Huang *et al.*, 1994) together with studies using chimeric mice generated subsequent to DNA inoculation (Doe *et al.*, 1996) suggested that antigen transfer between somatic cells and bone marrow-derived APC may be important for CTL priming.

A few factors may account for these discrepancies: the heterogeneity of the models employed regarding the route and dose of vaccination, the type of antigen and particularly the presence or absence of targeting signals. Furthermore, it is likely that both mechanisms co-exist to a certain degree.

1.4 Advantages of DNA vaccines over current vaccines

DNA vaccination provides many advantages over current vaccines that use live-attenuated pathogens, protein- or peptide-based subunit vaccines or killed micro-organisms. As observed following live infection, DNA vaccination results in stimulation of both the cellular and the humoral arm of the immune system, whereas subunit vaccines or killed micro-organisms mainly induce antibody responses. In addition, it is possible to tailor the immune response to either a strong humoral or a strong cellular response depending on the route of delivery and the costimulatory molecules administered with the plasmid. Several studies have demonstrated that DNA vaccines can successfully induce immune responses in neonates, observing minimal interference from maternal antibodies (Monteil *et al.*, 1996; Fischer *et al.*, 2003; Hassett *et al.*, 1997; Van Loock *et al.*, 2004; Bot and Bona, 2002). In addition, they may overcome immaturity of the neonatal immune system due to the presence of CpG motifs (Bot and Bona, 2002). Another advantage of DNA vaccines includes safety, with no evidence of injection site reactions which are often observed with subunit or killed vaccines mixed in adjuvants, no risk of reversion to virulence which might occur with live-attenuated vaccines and no need to culture dangerous pathogens. Despite the initial concern that plasmid DNA may cause tumour genesis, integrate into the host chromosome (Nichols *et al.*, 1995) or induce anti-DNA auto-immunity (Donnelly *et al.*, 1997), yet little evidence has supported these phenomenon. Indeed, the rate of integration of plasmid DNA into the host chromosome appears to be much lower than the spontaneous mutation rate for mammalian genomes (Nichols *et al.*, 1995; Martin *et al.*, 1999) and the initiation of nucleic acid auto-immunity is not supported by data obtained in mice and cattle. In mice, anti-DNA antibodies to mammalian DNA could not be observed unless BSA or Freund's adjuvant were co-administered to generate an antibody response (Gilkeson *et al.*, 1993, 1996), whereas in cattle, no anti-DNA response could be observed for up to 8 months following 5 immunizations with 1 mg of plasmid DNA (Van Drunen Little-van den Hurk *et al.*, 1998). Moreover, the results of all clinical studies reported so far indicate that DNA vaccines appear to be safe and well tolerated in humans (reviewed by Donnelly *et al.*, 2003). Furthermore, DNA vaccines are easy to produce. They are also considered more temperature-stable than conventional vaccines, removing the need for a cold chain which is a very costly and difficult issue. This is also of great significance in veterinary medicine, especially in developing countries.

1.5 Strategies applied to enhance the efficacy of DNA vaccines in pigs

Despite the numerous advantages of DNA vaccines, they are often hampered by poor efficacy, especially in large animals and man. Several advances have therefore been made to enhance the potency of these vaccines, and there still exists room for improvement. In the present review, we mainly focus our discussion on approaches that have been employed in pigs, since they are one of the most attractive large animal models. This is because they are easy to handle and the costs of obtaining and housing them are substantially lower compared to other large animals. Furthermore, as swine diseases have a large economical impact, most of their pathogens are well characterized. A third reason for the popularity of pigs in vaccination studies is that they are physiologically very similar to man, which allows them to be used as a model. We will also include relevant data obtained in mice if necessary to highlight areas that require further investigation.

1.5.1 Expression optimization

As DNA vaccination requires the *in vivo* expression of the encoded antigen, it is clear that the level of protein expression can have a direct impact on the magnitude of the induced response (Montgomery *et al.*, 1993). Therefore, plasmids should be optimized to enable efficient protein expression. There is a rationale behind engineering plasmids that express high levels of antigen which involves the inserted gene sequence as well as vector elements. Elements involved in expression are discussed below.

The inserted sequence

Recognition of the AUG translation initiation codon within mRNA is influenced by its flanking sequences. By analysing the expression of several hundred mammalian genes, the 'Kozak' consensus sequence ($^{-6}$ GCCA/GCCAUGG $^{+4}$) has been described for translational initiation in mammals (Kozak, 1987). Hereby, especially the presence of a purine (G/A) at -3 and a G at the +4 position has been suggested to be important for the efficiency of translational initiation (Kozak, 1997). As prokaryotic genes do not possess Kozak sequences, introducing them has been suggested to increase their expression level in eukaryotic cells (Garmory *et al.*, 2003).

While the genetic code is universal to all life as we know it, the efficiency with which an organism uses a particular codon varies dependent upon which tRNA pools are rate limiting in that particular species (Grosjean and Fiers, 1982). In general, differences in tRNA availability between species become larger as they are diverged earlier during evolution. Therefore, nucleotide sequences of many pathogens may not be optimal for translation in

mammalian cells. Studies in mice have reported accelerated seroconversion, increases of up to 100-fold in antibody responses (Narum *et al.*, 2001; Vinner *et al.*, 1999; Deml *et al.*, 2001) and improved CTL responses (Andre *et al.*, 1998; Deml *et al.*, 2001) by optimizing the gene sequence for translation in the target organism. Besides by improving tRNA availability, codon-optimization can also increase expression by avoiding secondary structures, internal splice sites and cis-acting sequences in mRNA, which all can prevent protein synthesis.

So far, there are no published studies addressing these strategies in pigs. However, results obtained in mice suggest that they could represent a valuable contribution.

Vector elements

The cytomegalovirus immediate early (CMV IE) promoter has been shown to drive the highest level of transgene expression in various tissues when compared with other promoters (Cheng *et al.*, 1993; Manthorpe *et al.*, 1993; Galvin *et al.*, 2000) and for this reason has been selected to drive expression of most DNA vaccines. Furthermore, it has been demonstrated that inclusion of the first intron (intron A) of the CMV IE gene further improved expression by the CMV IE promoter (Chapman *et al.*, 1991; Van Drunen Littel-van den Hurk *et al.*, 1999; Wang *et al.*, 2006). This beneficial effect has been credited to binding of transcription factors (Chapman *et al.*, 1991) and to an enhanced rate of RNA polyadenylation and/or nuclear export associated with splicing (Huang and Gorman, 1990).

Much less attention has been given to the sequences and genetic elements included in the 3' sequences that flank the gene of interest. This region contains not just the polyadenylation sequences required for stabilization of mRNA and transcription termination, but may also contain enhancer sequences that can dramatically alter the level of gene expression. Most DNA vaccines contain the 3' region derived from the bovine growth hormone (BGH) or the SV40 polyadenylation site (polyA). A few studies demonstrated higher expression levels when the BGH polyA was used for transcriptional termination *in vitro* (Montgomery *et al.*, 1993) as well as *in vivo* in mice (Norman *et al.*, 1997), although in a study by Xu *et al.* (2001), the activity of both BGH and SV40 polyA appeared to be cell line and tissue depended. A more recent study in mice demonstrated that the use of the HBV polyA significantly increased antigen expression and immune induction when compared with the BGH polyA, suggesting that further optimization of polyA sequences may be valuable (Zinckgraf and Silbart, 2003).

Self-replicating vectors

Another strategy that has been employed to obtain high level protein expression is the use of self-replicating alphavirus DNA-based vectors (Karlsson and Liljestrom, 2004; Nordstrom *et al.*, 2005). Unlike conventional DNA vaccines in which an RNA polymerase II dependent

promoter drives transcription of a heterologous gene, these vectors utilize the eukaryotic RNA polymerase II dependent promoter to transcribe a self-replicating alphavirus RNA transcript (replicon). This replicon is essentially an alphavirus genome containing the sequences necessary for replication but having the structural genes replaced by the heterologous gene. Consequently, virus formation does not occur. Due to the presence of the replicase genes, the mRNA will directly be replicated in the cytoplasm leading to an estimated 200,000 copies per cell, resulting in an extremely high protein expression (Lundstrom, 2000). However, this is only transient as the cytoplasmic replication eventually triggers apoptosis. Additional factors that may contribute to the enhanced immunogenicity of these vectors compared to conventional DNA vaccines include the ability of replicating dsRNA to stimulate innate antiviral pathways in the transfected cell (Leitner *et al.*, 2003) and the release of antigenic material for cross-presentation following apoptosis (Leitner *et al.*, 2004). An extra advantage is that the induction of apoptosis alleviates the concern of chromosomal integration. Self-replicating DNA vectors have been described based on the Semliki forest virus (Berglund *et al.*, 1996) and the Sindbis virus replicon (Dubensky *et al.*, 1996).

Dufour and de Boissésón (2003) were the first to evaluate the potential benefits of using a Sindbis virus DNA-based expression vector (pSIN) in pigs. They compared the efficacy of three pSIN plasmids encoding the gB, gC and gD major envelope glycoproteins of Pseudorabies virus (PRV) to three pcDNA3 vectors encoding the same antigens. Two intramuscular injections of the same plasmid copy numbers of both vectors types only induced weak antibody and T cell responses and resulted in equivalent protective immunity against PRV. Consequently, a greater efficiency of the pSIN vectors could not be observed. Those results were in contrast to a study by Hariharan and colleagues (1998) in mice in which pSIN was superior to a conventional plasmid (pCI) in the induction of all tested immune parameters. Moreover, Hariharan *et al* demonstrated that 100- to 1,000-fold lower doses of pSIN were needed to obtain the same responses as achieved with the conventional plasmid. A dose-dependent analysis of the PRV gB, gC and gD encoding pSIN plasmids in pigs was done by Dory *et al* (2005b) in a single shot immunization protocol. Pigs were immunized intramuscularly either with 340 µg of each of the pSIN plasmids corresponding to the quantity used by Dufour and de Boissésón (2003), with 68 µg of each plasmid (5-fold lower dose) or with 13 µg of each plasmid (25-fold lower dose). Although the induction of humoral immunity was dose-dependent, effective CTL responses and similar clinical protection were achieved with all three plasmid doses. These data indicated that also in pigs pSIN-based DNA vaccination allows for considerable reduction of the plasmid DNA dose. This is not only

important from an economical point of view but also regarding safety. More recently, the efficacy of a Semliki forest virus DNA-based vector (pSFV) was demonstrated in pigs (Li *et al.*, 2006). In that study, intramuscular immunization of pigs with 600 µg of pSFV encoding the E2 glycoprotein of classical swine fever virus resulted in detectable antibody responses and conferred complete protection against lethal challenge.

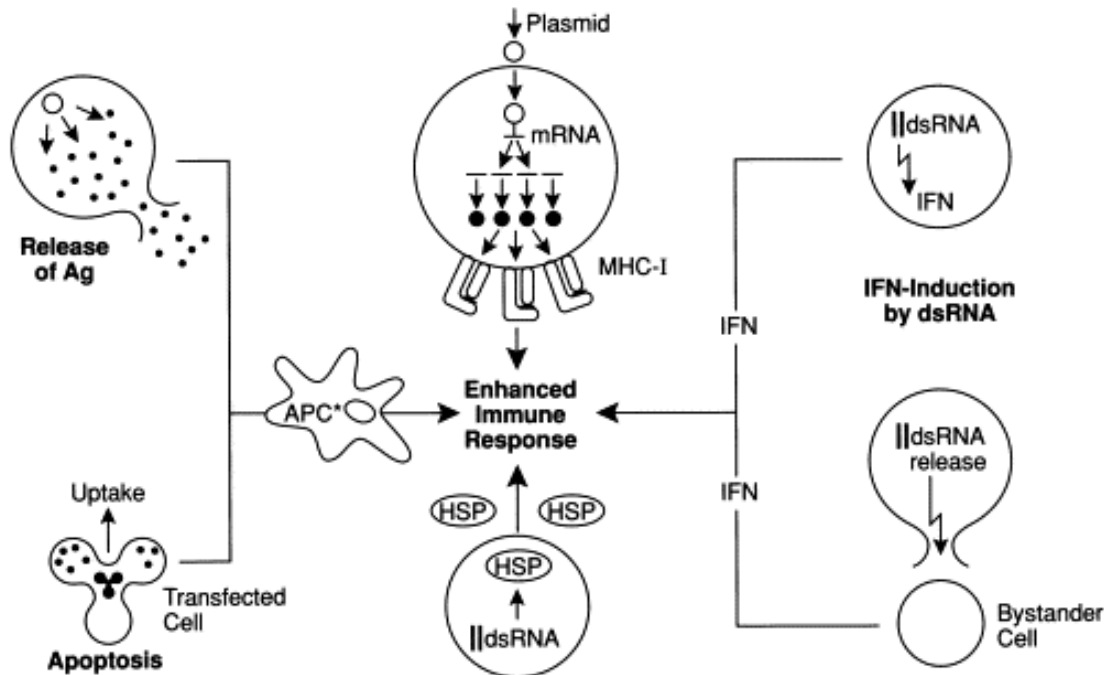


Figure 4. Potential factors contributing to the high immunogenicity of self-replicating genetic vaccines. (Starting in the upper centre and moving clockwise). Accumulation of antigen in the transfected cell can result in highly efficient MHC-I-loading. A number of ‘danger signals’ may be generated such as IFN production and IFN release from infected cells resulting from the presence of dsRNA. IFN may also be produced by bystander cells in response to dsRNA released from lysed transfected cells. Heat shock proteins (HSP) have also been shown to be produced in response to the presence of dsRNA. Ingestion of antigen-loaded apoptotic cells by APCs can also result in the elicitation of powerful immune responses. Finally, the local release of large amounts of antigen at the site of injection by transfected cells may be fed into resident APC. Adapted from Leitner *et al.*, 2000.

1.5.2 DNA delivery

In the past years, it has become apparent that the way a DNA vaccine is delivered may affect the type of response generated. It appears likely that both the site and method of inoculation may affect the magnitude and type of the induced response. In most studies concerning DNA vaccination of pigs, plasmid DNA has been delivered by the intramuscular (IM) or intradermal (ID) route, either by needle injection (Table 1), via a “gene gun” (Table 2) or jet injector (Table 3).

Intramuscular versus intradermal injection

Needle injection simply involves the direct injection of plasmid DNA dissolved in water, saline or a formulation (as later discussed). Following needle injection, plasmids can be actively taken up by cells in the injected area, but only few cells are directly transfected (Raz *et al.*, 1994; Davis *et al.*, 1993; 1995). Contradicting findings were observed when the efficacy of ID and IM needle injection was compared in pigs (Table 1). Using a cocktail DNA vaccine encoding pseudorabies virus (PRV) gB, gC and gD, van Rooij *et al* (1998) observed significantly stronger antibody responses and cell-mediated immunity, and better protection against challenge infection by ID than by IM injection. Their data are in line with results obtained in cats and cattle (Van Drunen Little-van den Hurk *et al.*, 1998; Osorio *et al.*, 1999) and may reflect the better immune surveillance function of the skin as compared to muscle (as earlier discussed). However, in another PRV trial in pigs, partial protection against PRV challenge could only be observed following IM needle injection. Furthermore, the IM injection was more efficient in the induction of an antibody response than ID needle injection (Gerds *et al.*, 1997). These opposite findings are likely a result from the fact that not only the site and method of inoculation, but also the encoded antigen determine the outcome of the immunization. Whereas van Rooij and colleagues (1998) used a gB, gC, gD cocktail DNA vaccine, only the gC plasmid was used by Gerds *et al* (1997).

Gene gun delivery

In gene gun-mediated delivery, gold particles coated with plasmid DNA are propelled by helium or CO₂ pressure into the skin. Hereby, plasmid DNA is directly deposited into cells of the epidermis and dermis, directly transfecting up to 20% of the cells of the target area (Williams *et al.*, 1991). Although only a small portion of these cells are DC, tissue stress resulting from the blast leads to a massive activation of DC in the target area, inducing them to migrate to draining lymph nodes where they provide necessary signals for effective T cell activation (Porgador *et al.*, 1998). While needle immunization generally requires large amounts of plasmid DNA, gene gun immunization requires approximately 100- to 1000- fold less DNA to induce comparable antibody responses as achieved by needle injection. Furthermore, gene gun immunization differs strikingly from needle injection in terms of the type of immune response induced. While IM or ID injection generally result in a Th1-like response, a predominantly Th2-like response is induced by gene gun immunization (Barry and Johnston, 1997; Feltquate *et al.*, 1997). Although the exact reason remains unclear, these differences were initially attributed to the low amount of plasmid DNA used with gene gun immunization, containing 'not enough' Th1-modulating CpG motifs. However, more recent

Table 1. Summary of DNA vaccines tested in pigs by needle injection.

Pathogen ^a	Antigen ^b	Route ^c	Number of immunizations and dose	Ab ^d	CMI ^e	Protection ^f	Reference
F4 ⁺ ETEC	FaeG	IM	3 x 600 µg	+	±	ND	Verfaillie <i>et al.</i> , 2004
PRV	gB + gC+ gD	IM or ID	3 x, 400 µg each	ID > IM	ID > IM	ID > IM > controls	van Rooij <i>et al.</i> , 1998
PRV	gC or gD gC	IM IM or ID	4 x 50 µg 3 x 1, 10 or 50 µg	gC > gD IM > ID; 50 > 10 > 1 µg	ND ND	gD: / ; gC: partial ID: /; IM: partial	Gerdts <i>et al.</i> , 1997
PRV	gB + gD	IM	1 x 200 µg each	+	ND	Partial	Somasundaram <i>et al.</i> , 1999
PRV	gD	IM	1 or 2 x 400 µg	In absence of Mat Ab	ND	/	Monteil <i>et al.</i> , 1996
FMDV	P1-2A3C3D	IM + ID	3 x 300 µg	+	±	Partial	Cedillo-Baron <i>et al.</i> , 2001
PRRSV	GP ₅	IM + ID	3 x 100 µg	+	+	Partial	Pirzadeh and Dea, 1998
<i>Taenia solium</i>	cC1	IM	2 x 500 µg	ND	ND	Partial	Wang <i>et al.</i> , 2003b

^a ETEC: enterotoxigenic *E. coli*, PRV: pseudorabies virus, FMDV: foot and mouth disease virus, PRRSV: porcine reproductive and respiratory syndrome virus

^b g/GP: glycoprotein, P1-2A: FMDV structural protein precursor, 3C3D: FMDV non-structural proteins

^c IM: intramuscular, ID: intradermal

^d Ab: antibody response, >: better than, +: induced, ND: not determined

^e CMI: cell-mediated immunity, ±: few animals

^f /: not induced

studies demonstrate that the gene gun bombardment process itself induces Th2-balancing ‘danger’ signals which can not be overwritten by CpG motifs present in the plasmid backbone (Liu *et al.*, 2005; Weiss *et al.*, 2002).

Haynes and colleagues (1996) were the first to describe the efficacy of the gene gun in pigs. They noted the induction of strong protective levels of immune responses with as little as 3 µg of plasmid DNA. Soon thereafter, Fuller *et al* (1997) described that gene gun delivery of three 0.5-1.5 doses of a hepatitis B DNA vaccine induced protective antibody responses comparable to those observed following three doses of a licensed recombinant protein vaccine. Similarly, gene gun administration of two 9 µg doses of an influenza DNA vaccine elicited similar protection in swine as compared to a commercially available inactivated whole-virus vaccine (Macklin *et al.*, 1998). Furthermore, gene gun administration of two 2-5 µg doses of a DNA vaccine against foot and mouth disease has resulted in partial (Beard *et al.*, 1999; Benvenisti *et al.*, 2001) or complete protection (Wong *et al.*, 2000) against viral challenge. In a study by Barfoed *et al* (2004a), gene gun administration of a porcine respiratory and reproductive syndrome virus (PRRSV) DNA vaccine induced an antibody response that was higher and appeared earlier than the antibody response seen following IM/ID needle injection of a one order higher dose and although gene gun immunization did not protect the pigs against PRRSV challenge infection, it did result in an earlier appearance of virus neutralizing antibodies. In another trial, gene gun administration of two 60 µg doses of a PRRSV DNA vaccine failed to induce a detectable antibody response or virus-specific lymphocyte proliferation. Nevertheless, it did reduce viral shedding and accelerated viral clearance (Rompato *et al* 2006). Finally, although gene gun immunization against F4⁺ ETEC of an FaeG DNA vaccine was inferior to IM injection in inducing serum antibodies, it did induce stronger FaeG-specific lymphocyte proliferation following 3 administrations of a 60-fold lower dose (Verfaillie *et al.*, 2004).

Jet injection

DNA vaccines can also be delivered by needle-free liquid jet-injectors. With these injectors, liquid is shot directly into the skin by applying high-pressure onto the jet stream. The vaccine can be delivered intradermally, subcutaneously or intramuscularly depending on the force and distance of the jet stream from the skin surface. The fluid is spread throughout the tissue because it follows the path of least resistance, with some liquid penetrating cell membranes. This method allows the plasmid to directly transfect some cells, thereby improving the transfection efficiency (reviewed by Van drunen Little-van den Hurk *et al.*, 2004).

Table 2. Summary of DNA vaccines tested in pigs by gene gun

Pathogen ^a	Antigen ^b	Number of immunizations and dose	Ab ^c	CMI ^d	Protection	Comparison with conventional vaccine or alternative delivery route ^e	Reference
Hepatitis B	HBsAg	2 x 3 µg	+	ND	ND	ND	Haynes <i>et al.</i> , 1996
Hepatitis B	HBsAg	3 x 0.5-1.5 µg	+	ND	ND	ND	Fuller <i>et al.</i> , 1997
Influenza	HA	2 x 7.5 µg	+	ND	Partial	Ab response <, protection = inactivated virus	Macklin <i>et al.</i> , 1998
	NP	2 x 7.5 µg	+	ND	/	Ab response <, protection < inactivated virus	
FMDV	entire FMDV genome with mutation in cell binding site	2 x 3 µg	> empty capsid	ND	+	Ab response <, protection < inactivated virus	Beard <i>et al.</i> , 1999
	Empty capsid	2 x 3 µg	< entire genome	ND	/	Ab response <, protection < inactivated virus	
FMDV	F1-sclgG	2 x 2 µg	+	+	+	Ab response <, CMI >, protection = commercial vaccine	Wong <i>et al.</i> , 2000
FMDV	Serotype O1 P12A3CD	2 x 5 µg	/	ND	Partial	Protection < commercial vaccine	Benvenisti <i>et al.</i> , 2001
	O1 VP1, Asia VP1, O1 C3	2 x 5 µg	/	ND	Partial, < P12A3CD DNA	Protection < commercial vaccine	
PRRSV	NP	4 x 32 µg	+	ND	ND	Ab response > IM+ID injection	Barfoed <i>et al.</i> , 2004
PRRSV	N	2 x 60 µg	/	/	Partial	ND	Rompato <i>et al.</i> , 2006
F4 ⁺ ETEC	FaeG	3 x 10 µg	/	+	ND	CMI >, Ab response < IM injection	Verfaillie <i>et al.</i> , 2004

^a FMDV : foot and mouth disease virus, PRRSV : porcine respiratory and reproductive syndrome virus, ETEC: enterotoxigenic *E. coli*.

^b HBsAg: hepatitis B surface antigen, HA: hemagglutinin, NP: nucleoprotein, F1-sclgG: epitopes 141-160 and 200-213 of FMDV viral protein 1 fused to swine IgG heavy chain, P12A3CD: the FMDV structural proteins P1 and 2A together with the non-structural proteins 3C and 3D in one reading frame, VP1: viral protein 1

^c Ab: antibody response, +: induced, >: better than, <: smaller than, /: not induced

^d CMI: cell-mediated immunity, ND: not determined

^e =: comparable to

Table 3. Summary of DNA vaccines tested in pigs by jet injection

Pathogen ^a	Antigen ^b	Number of immunizations and dose	Route ^c	Injector	Ab ^d	CMI ^e	Protection ^f	Comparison with conventional vaccine or alternative delivery route ^g	Reference
Hepatitis B	HBsAg	2 x 500 µg	ID	BioJect	+	ND	ND	Ab response = ID needle injection (in absence of electroporation)	Babiuk <i>et al.</i> , 2003a
PRV	gC	3 x 1,10 or 50µg	ID	IDAL	50 > 10 > 1 µg	50µg: + 1 or 10 µg: ND	Partial	Ab response > IM and ID needle injection, protection > ID needle injection, = IM needle injection CMI < attenuated PRV	Gerdts <i>et al</i> 1997
PRV	gB+gC+gD+gE gC	3 x 12.5 µg each 3 x 50 µg	ID ID	IDAL	+, > gC +	ND ND	Partial /	ND ND	Gerdts <i>et al.</i> , 1999
	gB+gC+gD	3 x 15 µg each	ID	IDAL	+	ND	Partial	protection > adjuvanted inactivated vaccine, < attenuated live vaccine	
PRV	gB+gC+gD	3 x 400 µg each	ID	Pigjet TM	+	+	Partial	CMI <, Ab response <, protection < ID needle injection	Van Rooij <i>et al.</i> , 1998
CSFV	Gp55	2 x 50, 100 or 200 µg or 1 x 200 µg	ID	Panjet	+, 2 x 200µg dose best	ND	+	Ab response > intra-lymph node or gene gun inoculation	Andrew <i>et al.</i> , 2000
-	hGH	1 or 2 x 0.5 or 0.05 mg/kg	IM or sc	Medi-Jector TM	+	ND	ND	Ab response > needle injection	Anwer <i>et al.</i> , 1999

^aPRV: pseudorabies virus, CSFV: classical swine fever virus^bHBsAg: Hepatitis B surface antigen, g/gp: glycoprotein, hGH: human growth hormone^cID: intradermal, IM: intramuscular, sc: subcutaneous^dAb: antibody response, +: induced, > better than^eCMI: cell-mediated immunity, ND: not determined^f/: not induced^g=: comparable to, < smaller than

Contradicting findings were obtained when these needle-free liquid jet-injectors were tested in pigs, which may be due to the fact that distinct types of jet-injectors were used in the different studies. Babiuk *et al* (2003a) demonstrated that ID needle-free delivery of plasmid DNA using the BioJect resulted in higher transgene expression levels than ID needle injection. However, in the absence of electroporation (see below), BioJect delivery did not induce a clearly stronger Ab response (Babiuk *et al.*, 2003a). In another trial, Van Rooij *et al* (1998) compared the efficacy of the needle-free PigjetTM injector and ID needle injection to induce an immune response against a cocktail DNA vaccine encoding PRV gB, gC and gD. In that study, the needle-free injector was not superior to needle injection in inducing antibody responses, cell-mediated immunity or protection against challenge infection. In a similar experiment, Gerdts *et al* (1997) vaccinated pigs with a DNA vaccine encoding PRV gC by either ID or IM needle injection, or by the needle-free IDAL injector. In contrast to the findings of Van Rooij, the IDAL injector was most efficient in inducing neutralizing antibody responses and elicited partial protection against challenge infection. Moreover, although jet injection of the PRV cocktail DNA vaccine was less effective than a live-attenuated vaccine, it was superior to a commercially available inactivated vaccine (Gerdts *et al.*, 1999). In agreement with the results of Gerdts *et al*, ID delivery of CSFV gp55 DNA via the Panjet appeared to be superior for the induction of a virus neutralizing Ab response than intralymph node injection or gene gun delivery (Andrew *et al.*, 2000).

Besides ID, needle-free delivery was also tested in pigs by the IM and the subcutaneous route using the Medi-JectorTM (Anwer *et al.*, 1999). The needle-free delivery by both routes was superior to needle injection for the induction of antibody responses, resulting in 3- to 4-fold higher serum IgG titers.

The IDAL jet-injector is commercially available and is used to deliver conventional vaccines against PRV and PRRSV in pigs.

Electroporation

Another promising means to improve the potency of DNA vaccines is electroporation. This physical process exposes the target tissue to brief electric pulses that induce temporary and reversible pores in cell membranes such as the plasma, nuclear and organelle membranes (Morgan and Day, 1995). Electroporation has been shown to increase both the number of transfected cells and the number of plasmids taken up by each cell (Aihara and Miyazaki, 1998). Consequently, gene expression levels can be improved by as much as 1 or 2 orders of magnitude over plasmid injection alone (Aihara and Miyazaki, 1998; Mathiesen, 1999). The increase in gene expression (Widera *et al.*, 2000), but also tissue damage and ensuing

inflammation caused by electroporation have been suggested to be important mechanisms for enhancing immune responses to DNA vaccines (Babiuk *et al.*, 2002; 2004). Although electroporation has most often been applied to muscle and skin, successful electroporation of various other tissues, such as lungs (Dean *et al.*, 2003), cornea (Blair-Parks *et al.*, 2002) and spinal cord (Lin *et al.*, 2002), has been described. Furthermore, electroporation has been shown to enhance plasmid expression and plasmid induced responses in a variety of animal models, including pigs (Table 4). In pigs, electroporation was shown to enhance gene expression up to 83-fold following ID injection of plasmid DNA (Drabick *et al.*, 2001). Importantly, these investigators also demonstrated that a non-invasive plate electrode is equally effective as a penetrating needle array electrode in enhancing gene expression. The efficacy of needle-free electroporation in pigs was also evaluated by Babiuk *et al.* (2003a, 2003b). They reported a 1 to 2 log increase in transgene expression and concomitantly, a 1 to 1.5 log higher antibody titer following 2 DNA vaccinations and a protein boost when needle-free electroporation was applied to ID needle injection or needle-free delivery (Babiuk *et al.*, 2003a, 2003b). The same investigators also evaluated the impact of electroporation on IM immunization in pigs, using plasmids encoding two different genes (bovine herpesvirus gD and hepatitis B surface antigen) and two different types of electrodes, a single-needle and a six-needle electrode (Babiuk *et al.*, 2002). Electroporation resulted in a 100- and a 1000-fold increase in transgene expression for the single- and six-needle electrode, respectively. Furthermore, an up to 1 and up to 2 log increase in antibody titer could be observed following two DNA vaccinations when using a single- or a six-needle electrode, respectively. So, although encouraging results were obtained with the less-invasive needle-free electroporation strategies, muscle electroporation currently remains more effective in eliciting immune responses to DNA vaccines than any other approach (Babiuk *et al.*, 2002, 2003a, 2003b).

Table 4. The influence of electroporation on DNA vaccines tested in pigs

Pathogen ^a	Antigen ^b	Method ^c	Number of immunizations and dose	Type of electrode	Gene expression	Ab ^d	CMI ^e	Reference
Hepatitis B	Luciferase	ID needle injection	1 x 50, 100, 200 or 300 µg	Penetrating (2 rows of seven 7-mm pins)	83-fold increase at 50 µg dose, decreasing on with increasing DNA dose	ND	ND	Drabick <i>et al.</i> , 2001
		ID needle injection	100 µg	Penetrating non-invasive	26-fold increase 26-fold increase	ND	ND	
	Luciferase	ID needle injection	100 µg	non-invasive	2 log increase	ND	ND	Babiuk <i>et al.</i> , 2003a, 2003b
		ID needle free delivery (BioJect)	100 µg	non-invasive	1 log increase	ND	ND	
	HBsAg	ID needle injection	2 x 500 µg	non-invasive	ND	1.5 log higher (after protein boost)	ND	Babiuk <i>et al.</i> , 2003a, 2003b
		ID needle free delivery (BioJect)	2 x 500 µg	non-invasive	ND	1log higher (after protein boost)	ND	
BHV, Hepatitis B	gD, HBsAg	IM needle injection	2 x 1 mg of gD or HBsAg or both	single-needle	2 log increase	Up to 1 log higher	Higher proliferation	Babiuk <i>et al.</i> , 2002
				six-needle	3 log increase	Up to 2 log higher	Higher proliferation, Th1-biasing	

^a BHV: bovine herpes virus.^b g: glycoprotein, HBsAg: Hepatitis B surface antigen.^c ID: intradermal, IM: intramuscular^d Ab: antibody response, ND: not determined^e CMI: cell-mediated immunity

1.5.3 Adjuvants

Several approaches undertaken to enhance the potency of DNA vaccines have focused on the use of adjuvants. Adjuvants can be broadly separated into genetic and conventional adjuvants, based on their origin. Genetic adjuvants are expression vectors encoding cytokines, chemokines, costimulatory molecules or their ligands, or other molecules that can modulate immune responses when administered with a vaccine antigen. Conventional adjuvants are chemical compounds that enhance, prolong, or modulate antigen-specific immune responses. The use of an appropriate adjuvant can be pivotal in optimizing not only the magnitude but also the nature of the induced response. Moreover, DNA vaccines themselves possess their own intrinsic adjuvant activity through CpG motifs (Sato *et al.*, 1996).

CpG motifs

Co-administration of synthetic CpG oligodeoxynucleotides (ODN) has been shown to activate the immune system and to augment the immunogenicity of protein-based vaccines (Davis *et al.*, 1998, McCluskie *et al.*, 2002, Klinman, 2006). However, contradicting results were obtained when the influence of CpG motifs on DNA vaccines was evaluated. In mice, it was demonstrated that the response elicited by suboptimal amounts of plasmid DNA could be boosted by co-administration of free CpG ODN (Klinman *et al.*, 2004). In line with these results, Dory *et al* (2005a) demonstrated that mixing of a porcine-specific CpG ODN to plasmids encoding PRV gB, gC and gD increased PRV-specific humoral and cellular immune responses and resulted in better clinical protection of pigs against a PRV challenge infection. On the contrary, other studies in mice, horses and pigs observed no effect or even a reduction (Cantlon *et al.*, 2000; Van Rooij *et al.*, 2002; Weeratna *et al.*, 1998). Interestingly, Weeratna *et al* (1998) reported a dose dependent reduction of the immune response when CpG ODN were co-administered with high levels of a DNA vaccine. It was hypothesized that this reduction in immune response was due to competition between plasmid DNA and CpG ODN for entry into the target cells. Thus, inclusion of the CpG motifs into the plasmid backbone is likely to be more beneficial. Indeed, several studies suggested that CpG motifs present in the plasmid backbone might contribute to the immunogenicity of DNA vaccines in outbred animals as well as in mice. For example, methylation of all cytidines in pcDNA3 abolished IFN- α secretion when the plasmid was incubated with porcine leukocytes (Magnusson *et al.*, 2001), whereas insertion of an optimal number of immunostimulatory CpG motifs into the plasmid backbone enhanced the induction of vaccine-specific immune responses in mice (Krieg *et al.*, 1998; Ma *et al.*, 2002; Kojima *et al.*, 2002). Similarly, a study in cattle using plasmids containing 0, 40 and 88 ruminant-specific CpG motifs showed a dose-dependent

increase in antigen-specific cellular immune responses. However, they did not observe a quantitative effect on humoral immunity (Pontarollo *et al.*, 2002).

So far, there are only few studies addressing this issue in large animals. Therefore additional, more comprehensive studies are necessary to completely establish the efficacy of CpG adjuvanting of DNA vaccines in large animals. For example, it remains to be determined whether different motifs can preferentially induce different types of immune response and thus can be used to direct the immune response differently. Furthermore, as CpG ODN have been described to have the most effect when small doses of plasmid DNA are used (Weeratna *et al.*, 1998), dose-response studies in outbred species need to be performed.

Genetic cytokine adjuvants

Genetic adjuvants encoding a large number of cytokines have been used to enhance the magnitude of the induced response and/or to modify the response towards either a strong Th1- or a strong Th2-type response. The studies performed in pigs using DNA encoded cytokines as adjuvants are summarized in Table 5.

A frequently tested genetic adjuvant is the GM-CSF gene. In mice, GM-CSF DNA has been shown to enhance humoral and CD4⁺ T cell responses, in most cases without shifting the balance between Th1 and Th2 type responses. This is likely a result of the effect of GM-CSF on APC. GM-CSF has been described not only as a growth and differentiation factor for APC but also as a chemo-attractant for these cells (Kaplan *et al.*, 1992). Indeed, plasmid encoded GM-CSF has been shown to recruit APC to the injection site and expand the number of APC in the draining lymph node in mice (Bowne *et al.*, 2000; Haddad *et al.*, 2000). In pigs, plasmid encoded GM-CSF has been shown to enhance neutralizing antibody titers (Andrew *et al.*, 2006; Cedillo-Baron *et al.*, 2001), IgG1 and IgG2 responses (Somasundaram *et al.*, 1999) and both Th1- and Th2-cell responses (Dufour *et al.*, 2000), and to result in enhanced protection against viral challenge (Somasundaram *et al.*, 1999, Andrew *et al.*, 2006). While the above mentioned studies have co-injected plasmid encoded GM-CSF with the DNA vaccine, several studies suggest that the time of cytokine DNA injection relative to the time of the DNA vaccine injection might be critical for obtaining a maximal adjuvant effect (Kusakabe *et al.*, 2000; Barouch *et al.*, 1998). However so far, there are no studies that examined the influence of the interval between GM-CSF administration and the DNA vaccination on its adjuvanticity.

Th1-inducing cytokine genes evaluated in pigs include IL-2, IL-12, IL-18 and IFN- γ . Plasmid encoded IL-2 has been shown to enhance the induction of virus specific cell-mediated immune responses following DNA vaccination against PRRSV (Rompato *et al.*,

2006) and FMDV (Wong *et al.*, 2002). Conversely, Somasundaram *et al* (1999) could not observe an adjuvant effect when IL-2 or IFN- γ were co-administered with a PRV DNA vaccine. Other investigators also failed to demonstrate significantly stronger immune responses or better protection against challenge infection by co-delivery of IFN- γ or IL-12 with a PRV DNA vaccine (Van Rooij *et al.*, 2002). The immunomodulatory effects of plasmid encoded IL-12 and IL-18 were evaluated on DNA vaccination of pigs against classical swine fever (Wienhold *et al.*, 2005). An earlier appearance of serum antibodies, a reduced B cell deficiency and a better protection against challenge infection were observed when IL-18 was co-administered with the DNA vaccine. In contrast, co-delivery of IL-12 reduced the antibody titer as well as protection against lethal challenge infection. Conversely, IL-12 DNA was shown to enhance the protective efficacy of a DNA vaccine against *Schistosoma japonicum* (Zhu *et al.*, 2004).

Studies evaluating the influence of Th2-type cytokine genes in pigs are restricted to IL-4 and IL-6. Rompato *et al* (2006) evaluated the influence of plasmid encoded IL-4 on the induction of cellular and humoral immunity following PRRSV DNA vaccination. Co-administration of IL-4 DNA had an inhibitory effect on the induction of cell-mediated immunity and did not enhance humoral immunity. The lack of a positive effect on antibody responses led the authors to hypothesize that IL-4 by itself may not have a critical role in the development of a Th2 immune response and that other Th2 cytokines, like IL-6 and IL-13 may be necessary for the generation of such responses in swine. However, also co-administration of plasmid encoded IL-6 with an influenza DNA vaccine did not significantly enhance serum antibody responses or protection from challenge infection (Larsen and Olsen, 2002). Immunomodulatory effects of the IL-13 gene remain to be tested.

As far as we know, plasmid encoded chemokines have not been tested in pigs. Therefore, they will not be discussed in this review.

Other genetic adjuvants

CD154 (CD40 Ligand) is expressed on NK cells, DC and activated lymphocytes. Its interaction with CD40 influences and sustains co-stimulatory signalling in APC which is essential for T cell dependent antibody responses. Co-delivery of the CD154 gene with a CSFV DNA vaccine in pigs resulted in an earlier appearance of serum antibodies, reduced B cell deficiency after infection and protection against a lethal CSFV challenge infection (Wienhold *et al.*, 2005).

Another means of improving the efficacy of DNA vaccines is by targeting antigen to APC. In this regard, targetting the B7 (CD80/86) costimulatory molecules on the surface of APC

Table 5. Influence of co-administration of genetic adjuvants on DNA vaccine efficacy in pigs.

Pathogen ^a	Antigen ^b	Adjuvant	Method and route ^c	Ab response ^d						CMI ^e				protection	Reference
				VN	Total IgG	IgG1	IgG2	IgA	IgM	CTL	Prolife-ration	Th1	Th2		
CSFV or Influenza	Gp55 or HA	GM-CSF IL-3	Needle free delivery (Panjet)	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	Andrew <i>et al.</i> , 2006
				+	ND	/	/	/	+	ND	ND	ND	ND	+	
FMDV	P1-2A3C3D	GM-CSF	IM + ID needle injection	+	+	ND	ND	ND	ND	ND	ND	ND	ND	+	Cedillo-Baron <i>et al.</i> , 2001
PRV	gB, gD	GM-CSF IL-2 IFN- γ	IM needle injection	/	+	+	+	ND	ND	ND	ND	ND	ND	+	Somasundaram <i>et al.</i> , 1999
				/	/	/	/	ND	ND	ND	ND	ND	ND	/	
				/	/	/	/	ND	ND	ND	ND	ND	ND	/	
PRV	gB, gC, gD	GM-CSF IFN- α	IM needle injection	ND	/	/	/	ND	ND	ND	ND	+	+	+	Dufour <i>et al.</i> , 2000
				ND	/	/	/	ND	ND	ND	ND	/	/	/	
PRRSV	NP	IL-2 IL-4	Gene gun	ND	-	ND	ND	ND	ND	ND	+	ND	ND	-	Rompato <i>et al.</i> , 2006
				ND	-	ND	ND	ND	ND	ND	/	ND	ND	-	
FMDV	VP1(141-160), VP1(200-213)	IL-2	IM needle injection	/	ND	ND	ND	ND	ND	ND	+	ND	ND	/	Wong <i>et al.</i> , 2002

^a CSFV: classical swine fever virus, FMDV: foot and mouth disease virus, PRV: pseudorabies virus, PRRSV: porcine reproductive and respiratory syndrome virus

^b HA: hemagglutinin, gp/g: glycoprotein, P1-2A3C3D: FMDV structural protein precursor P1-2A and the FMDV non-structural proteins 3C3D, NP: nucleoprotein, VP: viral protein

^c IM: intramuscular, ID: intradermal

^d Ab: antibody, VN: virus neutralizing antibodies, +: positive effect, /: no effect, -: negative effect, ND: not determined

^e CMI: cell-mediated immunity

Table 5. Influence of co-administration of genetic adjuvants on DNA vaccine efficacy in pigs. Continued.

Pathogen ^a	Antigen ^b	Adjuvant	Method and route ^c	Ab response ^d						CMI ^e				protection	Reference
				VN	Total / IgG	IgG1	IgG2	IgA	IgM	CTL	Prolife- ration	Th1	Th2		
PRV	gB, gD	IFN- γ	ID needle	/	ND	ND	ND	ND	ND	ND	/	ND	ND	+	Van Rooij <i>et al.</i> , 2002
		IL-12	injection	/	ND	ND	ND	ND	ND	ND		ND	ND	+	
CSFV	gp55	IL-12	IM needle injection	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	Wienhold <i>et al.</i> , 2005
		IL-18		+	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	
		CD154		+	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	
Schistosoma japonicum	SjC23	IL-12	IM needle injection	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	+	Zhu <i>et al.</i> , 2004
Influenza	HA	IL-6	Gene gun	/	/	ND	ND	/	ND	ND	ND	ND	ND	/	Larsen and Olsen, 2002
	OVA	CTLA4 (as a fusion with OVA)	Gene gun	NA	+	+	+	+	/	ND	ND	ND	ND		Tachedjian <i>et al.</i> , 2003

^a PRV: pseudorabies virus, CSFV: classical swine fever virus

^b g/gp: glycoprotein; HA: hemagglutinin; OVA: ovalbumin

^c ID: intradermal, IM: intramuscular

^d Ab: antibody response, VN: virus neutralizing antibodies; NA: not applicable; +: positive effect; /: no effect; -: negative effect; ND: not determined

^e CMI: cell-mediated immunity

via CTLA-4 antigen fusions has been reported to improve protein (Huang *et al.*, 2000) and DNA vaccines in mice (Boyle *et al.*, 1998; Deliyannis *et al.*, 2000), rabbits and monkeys (Jia *et al.*, 2006), sheep (Chaplin *et al.*, 1999; Drew *et al.*, 2001) and pigs (Tachedjian *et al.*, 2003). When pigs were immunized by gene gun with a DNA vector expressing a CTLA4-ovalbumine fusion protein, they developed enhanced ovalbumine-specific serum IgG, IgA, IgG1 and IgG2 responses compared to pigs immunized with the same vector expressing only ovalbumine (Tachedjian *et al.*, 2003).

Conventional adjuvants

There are only few published studies addressing the use of conventional adjuvants for DNA vaccines, suggesting that these adjuvants might not be as successful for DNA vaccines as they are for conventional vaccines. However, some conventional adjuvants, such as aluminum salts, (+)-N-(2-hydroxyethyl)-N,N-dimethyl-2-3-bis(tetradecyloxy)-1-propanedimium bromide and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DMRIE-DOPE) and dimethyldioctadecylammonium bromide (DDA) have shown a beneficial effect on DNA vaccination in large animals (Ulmer *et al.*, 1999; Fischer *et al.*, 2003b; van Rooij *et al.*, 2002). Co-delivery of DDA with a PRV DNA vaccine in pigs resulted in enhanced neutralizing antibody and T cell responses as well as reduced viral shedding following challenge, whereas co-delivery of plasmid encoded IL-12 or IFN- γ failed to do so (van Rooij *et al.*, 2002).

1.5.4 Formulations

DNA injection *in vivo* has been shown to result in extremely low transfection efficiencies and rapid clearance of the plasmids from the inoculation site (Kawabata *et al.*, 1995; Lew *et al.*, 1995). It follows that protecting plasmid DNA from extracellular degradation by improving its formulation should optimize DNA uptake. Unfortunately, only few studies have been performed on formulation of DNA vaccines in pigs as well as in other large animals. The most popular approaches in mice include the use of liposomes and polymers.

Liposomes are microscopic vesicles consisting of an aqueous core surrounded by a phospholipid bilayer. They are employed by many modern cell transfection techniques, such as Lipofectamine (Invitrogen) and Transfectam (Promega) to deliver DNA. Positively charged lipids assist in DNA complexation, can protect the DNA from nuclease degradation and aids in delivery to and association with the cell membrane. However, cellular toxicity associated with cationic lipids has been shown *in vitro* and *in vivo* (Filion and Phillips, 1997; Lappalainen *et al.*, 1994) and the future of this delivery technique in DNA vaccination remains uncertain (reviewed by Laddy and Weiner, 2006).

In addition to liposomes, polymer-based systems can be applied to protect plasmids from degradation. Polyvinylpyrrolidone (PVP) has been tested in both pigs and dogs by needle injection or needle-free delivery of a plasmid encoding human growth hormone (Anwer *et al.*, 1999). In that study, PVP formulation clearly increased antibody responses following needle injection as well as following needle-free delivery. One of the most promising polymer-based compounds used for delivery of DNA vaccines are cationic poly(lactide-co-glycolide) (PLG) microparticles (reviewed by Vajdy and O'Hagan, 2001; Singh *et al.*, 2006). The rationale for the use of these microparticles is based on their ability to be taken up by APC. After entering the phagocytic pathway, they produce antigen with access to both MHC class I and II pathways and are able to elicit antibody responses (Hedley *et al.*, 1998; Jones *et al.*, 1997; 1998). Furthermore, the PLG polymer has already been approved as a component of a number of drug delivery systems and has a long history of safe use in humans (Okada and Toguchi, 1995). While initial studies have encapsulated the plasmid within the PLG microparticles, a more recent technology involves the adsorption of plasmid DNA onto the outer surface of the microsphere (Vajdy and O'Hagan, 2001; Singh *et al.*, 2006). This technology has been shown to be immunogenic in humans and is in phase I clinical trials through the HIV Vaccine Trials Network (Laddy and Weiner, 2006).

1.5.5 Heterologous prime-boost immunization

The combination of DNA and conventional vaccines (heterologous prime –boost immunization) has been used in various ways to couple the benefits of both vaccine types, thereby providing stronger protective immunity. Generally, the immunization strategy begins with one or more doses of the first vaccine type (in most cases the DNA vaccine) (priming) followed by one or more doses of the other vaccine type (boosting). Heterologous prime –boost immunizations performed in pigs are presented in Table 6. A heterologous IM DNA prime (1x) – IM protein boost (2x) immunization was evaluated by Guo *et al* (2004) and compared to 3 IM protein immunizations. They observed a Th1-biased response (predominantly IgG2, strong lymphocyte proliferation) by the DNA prime – protein boost and a Th2-biased response (predominantly IgG1) following the 3 protein immunizations, with a longer duration of protection against *Taenia solium* cysticercosis using the heterologous protocol. Other investigators demonstrated that priming with DNA and boosting with recombinant virus was able to induce protective immunity in pigs against PRV (Dory *et al.*, 2006), post-weaning multisystemic wasting syndrome (Blanchard *et al.*, 2003) and classical swine fever (Hammond *et al.*, 2001).

Table 6. Influence of a heterologous prime - boost on DNA vaccine efficacy in pigs.

Pathogen ^a	Prime – boost vaccination ^b	Total/ VN ^c	IgG	IgG1	IgG2	IgA	CMI	Mucosal immunity	Protection	Reference
<i>Taenia solium</i>	IM DNA prime (1x) – IM protein boost (2x) IM protein immunization (3x)	ND	ND	+ ++	++ +	ND	++ +	ND	++ +	Guo <i>et al.</i> , 2004
HRV	Oral AttHRV prime (1x) – IM DNA boost (2x) IM DNA prime (2x) – oral AttHRV boost (1x) IM DNA immunization (3x) Oral AttHRV prime (1x) – IM empty vector (2x)	ND	++++ +++ +++ +++	ND	ND	++++ ++ + +++	ND	++++ ++ + +++	++++ ++ + +++	Yuan <i>et al.</i> , 2005
PRV	IM pSIN prime (1x) – IM ORFV boost (1x) / – IM ORFV boost (1x) IM pSIN prime (1x) – /	+++ + ++	ND	+++ + ++	+++ ++ ++	ND	+++ + ++	ND	+++ + ++	Dory <i>et al.</i> , 2006
PCV2	Trial 1 : IM DNA prime (2x) – IM protein boost (1x)	ND	ND	ND	ND	ND	ND	ND	+	Blanchard <i>et al.</i> , 2003
CSFV	Trial 2 IM DNA vaccination (2x) IM protein (2x) Trial 1: weaned pigs ID DNA (1x) – sc rPAV (1x) ID DNA (1x) – IM DNA (1x) ID DNA (1x) Sc rPAV (1x)		ND	ND	ND	ND	ND	ND	+ + ++ ++++ ++++ ++ +++	Hammond <i>et al.</i> , 2001
	Trial 2: suckling pigs ID DNA (1x) – sc rPAV (1x) Sc rPAV (1x)		ND	ND	ND	ND	ND	ND	++ +	

^a HRV: human rotavirus, PRV: pseudorabies virus, PCV2: porcine circovirus type 2, CSFV: classical swine fever virus

^b IM: intramuscular, ID: intradermal, sc: subcutaneous, AttHRV: attenuated human rotavirus, pSIN: Sindbis virus-based DNA vector, ORFV: Orf virus, parapoxvirus, rPAV: recombinant porcine adenovirus.

^c ND: not determined, +, ++, +++ and ++++ represent the strength of the response relative to the other strategies in the same experiment (+ < ++ < +++ < ++++)

Yuan and colleagues (2005) compared the IM DNA prime (2x) – oral boost strategy with oral priming using attenuated rotavirus and boosting with the DNA vaccine or 3 IM DNA immunizations in a human rotavirus infection model in pigs. Priming with the attenuated virus and boosting with DNA appeared to be the most effective strategy resulting in significant protection and the highest IgA response.

1.6 DNA vaccines for mucosal immunity

The mucosal surfaces are the primary site of transmission of most infectious diseases. Therefore vaccines which induce protective mucosal immunity will be highly effective in reducing morbidity and mortality. The vast majority of DNA vaccines are delivered parenterally, resulting in systemic immune responses which can react against pathogens in the body. However, they are generally not effective in inducing mucosal immunity and therefore can not prevent mucosal colonization or invasion. In contrast, mucosally delivered DNA vaccines might induce local mucosal as well as systemic immune responses. Furthermore, mucosal immunization at one site with antigen or attenuated microorganisms has been shown to induce immunity at distant mucosal sites as is the case for gut – mammary gland and for nasal mucosa – genital tract. However, several barriers are encountered before a mucosally administered DNA vaccine can reach the mucosa-associated lymphoid tissue. These include (i) an enzymatic barrier, caused by luminal secreted, luminal membrane bound and cytosolic enzymes; (ii) a diffusion barrier, by the mucus layer covering gastrointestinal epithelia; and (iii) an epithelial barrier. In this section, some of the strategies that have been explored to overcome these barriers, such as formulations, bacterial delivery and heterologous prime – boost immunizations will be discussed.

1.6.1 Formulations to improve mucosal DNA vaccination

When formulating DNA vaccines, it should be noted that effective mucosal immunization often correlates with the uptake of antigen at mucosal inductive sites, such as the Peyer's patches (PP) of the gut and nasal-associated lymphoid tissues. These inductive sites are equipped with specialized epithelial cells, called M cells. M cells are responsible for sampling of antigen from the lumen and for delivering it to antigen presenting cells underneath the epithelium.

PLG microparticles

Biodegradable PLG microparticles (< 10 µm) directly bind to and are subsequently taken up by M cells (Jepson *et al.*, 1993). They have been studied extensively as candidates for vaccine delivery via the oral and the nasal route.

Following initial studies on oral delivery of proteins, some groups evaluated the potential of PLG microparticles for oral delivery of DNA vaccines. Jones *et al* (1997) were the first to describe the successful induction of systemic and mucosal antibody responses following oral delivery of a DNA vaccine entrapped in PLG microparticles. Subsequently, two independent groups described the induction of protective mucosal immunity against rotavirus challenge in mice following oral immunization with plasmid DNA entrapped in PLG (Chen *et al.*, 1998; Herrmann *et al.*, 1999). Studies so far involve only rodent models and require relatively high doses in these species, indicating the need for significant improvements. Indeed, it appears clear that the approach of DNA encapsulating still has several limitations. These include plasmid damage by the encapsulation process and low encapsulation efficiency, resulting in a low DNA load and hereby limiting the total amount of DNA that can be administered. In addition, it seems likely that uptake of these microparticles into the GALT is insufficient to allow the successful development of oral vaccines (reviewed by O'Hagan *et al.*, 2004).

To overcome the limitations of the encapsulation process, a novel technology has been developed involving the adsorption of the plasmid onto the surface of cationic PLG microparticles. Important advantages of the adsorption approach include enhanced DNA stability and formulation loading efficiency, making this process much more amenable to commercial development. Intranasal administration of DNA vaccines adsorbed onto cationic PLG microparticles has shown some promise and outperformed naked DNA (Singh *et al.*, 2001).

Bioadhesive agents

Other promising delivery vehicles for mucosal DNA vaccines are chitosan micro- or nanoparticles. Chitosan is a polycation comprising copolymers of glucosamine and N-acetylglucosamine. It strongly interacts with negatively charged sialic acid residues in mucin (Lehr *et al.*, 1992), providing an intimate contact with the mucosal membrane and thereby increasing the halftime of clearance and promoting more efficient uptake (Alpar *et al.*, 2005). Chitosan can easily form complexes with DNA and it has been shown to effectively protect DNA from degradation (Mao *et al.*, 2001; Richardson *et al.*, 1999). It is also able to increase transcellular and paracellular transport across mucosal epithelia (Artursson *et al.*, 1994) and chitosan microparticles smaller than 10 µm have been demonstrated to be taken up by M cells

(van der Lubben *et al.*, 2001; 2002; Beier and Gebert, 1998). Several studies have demonstrated the successful use of chitosan as gene delivery vehicle via the oral (Chew *et al.*, 2003; Roy *et al.*, 1999) and the intranasal route (Kumar *et al.*, 2002; Iqbal *et al.*, 2003; Xu *et al.*, 2004) in mice. However, as far as we know, these particles have not yet been tested for mucosal delivery of DNA vaccines in large animals.

Another strategy that has been applied to improve DNA uptake through the NALT in mice includes the attachment of M cell ligands to the surface of DNA-polymer complexes. M cell ligands tested include the plant lectin Ulex europaeus agglutinin 1 (UEA-1) (Wang *et al.*, 2005) and reovirus $\sigma 1$ protein (Wang *et al.*, 2003a; Wu *et al.*, 2001). When DNA is conjugated to reovirus $\sigma 1$ protein via polylysine, the reovirus $\sigma 1$ protein can still bind the apical surface of nasal M cells (Wu *et al.*, 2001). Consequently, intranasal immunization with $\sigma 1$ -polylysine-DNA complexes induced prolonged mucosal IgA (Wu *et al.*, 2001) as well as cell-mediated immunity compared to naked DNA or polylysine complexed DNA (Wu *et al.*, 2001; Wang *et al.*, 2003a). Similarly, mice nasally immunized with UEA-1-polylysine-DNA complexes showed elevated systemic and mucosal antibody responses as well as elevated CTL responses compared to intranasal immunization with naked DNA (Wang *et al.*, 2005).

Although both $\sigma 1$ protein and UEA-1 are able to bind Peyer's patch M cells as well (Amerongen *et al.*, 1994; Clark *et al.*, 1995), their ability to improve the induction of mucosal immune responses following oral DNA vaccination has not yet been evaluated.

1.6.2 Live attenuated bacteria as delivery vehicles

Live bacteria have also been used to deliver DNA vaccines to mucosa-associated lymphoid tissues. With a few exceptions, these are virulence-attenuated strains of enteroinvasive bacteria such as Salmonella, Shigella or Listeria (reviewed by Schoen *et al.*, 2004), which cross the mucosal barrier via M cells and are then taken up by macrophages and DC at local sites (Neutra *et al.*, 1996; Siebers and Finlay, 1996; Sansonetti, 2002) or in systemic lymphoid tissues after spreading. After phagocytosis, the enteropathogens normally replicate within phagosomes (Salmonella spp) or escape from these phagosomes and replicate within the cytosol (Shigella and Listeria spp) (Schoen *et al.*, 2004). As a result of their metabolic attenuation, they only replicate for a few cycles whereafter they die and release the plasmid DNA inside the mammalian cell. After transport of the plasmid to the nucleus, the antigen becomes expressed and either processed and directly presented to T cells or released

Table 7. Vaccination of rodents or chicken against viral diseases using bacteria-mediated delivery of DNA vaccine plasmids

Pathogen ^a	Antigen ^b	Species	Attenuated bacterial carrier ^c	Delivery	Systemic response ^d		Mucosal response		Protection	Reference
					Ab	CMI	Ab	CMI		
HIV	gp120	Mouse	<i>S. flexneri</i>	Intranasal	ND	+	ND	ND	+	Shata and Hone., 2001 Vecino <i>et al.</i> , 2002
	gp120	Mouse	<i>S. flexneri</i> , <i>S. typhi</i> and <i>S. typhimurium</i>	Intranasal	+	+	+	ND	ND	
	Env	Mouse	<i>S. typhimurium</i>	Intragastric	ND	+	ND	+	ND	Shata <i>et al.</i> , 2001
	Gag	Mouse	<i>S. flexneri</i>	Intranasal	ND	+	ND	+	ND	Xu <i>et al.</i> , 2003
HBV	HbsAg	Mouse	<i>S. typhimurium</i>	Oral	+	+	ND	ND	ND	Woo <i>et al.</i> , 2001
HCV	NS3 region	Mouse	<i>S. typhimurium</i>	Oral	ND	+	ND	ND	+	Wedemeyer <i>et al.</i> , 2001
Measles virus	Fusion protein, hemagglutinin, nucleoprotein	Mouse	<i>S. flexneri</i> and <i>S. typhi</i>	Intranasal Intraperitoneal	+	+	ND	ND	ND	Fennelly <i>et al.</i> , 1999
	Hemagglutinin	Cotton rat	<i>S. flexneri</i> and <i>S. typhi</i>	Intranasal Intranasal	+	+	ND	ND	+	Pasetti <i>et al.</i> , 2003
HSV-2	gD, gB	Mouse	<i>S. typhimurium</i>	Oral	+	+	-	+	+	Flo <i>et al.</i> , 2001
MCMV	MCMV-BAC	Mouse	<i>E. coli</i>	Intramuscular, subcutaneous, intraperitoneal	+	ND	ND	ND	+	Cicin-Sain <i>et al.</i> , 2003
PRV	gD	Mouse	<i>E. coli</i>	Intramuscular	ND	+	ND	ND	+	Shiau <i>et al.</i> , 2001a
	gD, ProT	Mouse	<i>S. choleraesuis</i>	Oral	+	+	ND	ND	+	Shiau <i>et al.</i> 2001 b;
	ProT	Mouse	<i>S. choleraesuis</i>	Oral	+	+	+	ND	+	Shiau <i>et al.</i> , 2005
	gB	Mouse	<i>S. typhimurium</i>	Oral	+	+	+	+	(+)	Eo <i>et al.</i> , 2006
PRRSV	Gp5	Mouse	<i>S. typhimurium</i>	Oral	+	ND	ND	ND	ND	Jiang <i>et al.</i> , 2004
Influenza	hemagglutinin	Mouse	<i>S. flexneri</i>	Intranasal	+	+	+	ND	+	Vecino <i>et al.</i> , 2004
IBDV	VP2/4/3	Chicken	<i>S. typhimurium</i>	Oral	+	ND	ND	ND	+	Li <i>et al.</i> , 2006

^a HIV: human immunodeficiency virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HSV: herpes simplex virus-2; MCMV: murine cytomegalovirus; PRV: pseudorabies virus; PRRSV: porcine reproductive and respiratory syndrome virus; IBDV: infectious bursal disease virus

^b Gp/g: glycoprotein; HBsAg: Hepatitis B surface antigen; NS: non-structural; BAC: herpesvirus bacterial artificial chromosome; ProT: prothymosin α ; VP2/4/3: precursor polyprotein

^c *S. flexneri*: *Shigella flexneri*; *S. typhi*: *Salmonella typhi*; *S. typhimurium*: *Salmonella typhimurium*; *E. coli*: *Escherichia coli*; *S. choleraesuis*: *Salmonella choleraesuis*

^d ND: not determined, +: induced; -: not induced

Table 8. Vaccination of rodents against bacterial diseases using bacteria-mediated delivery of DNA vaccine plasmids

Pathogen	Antigen ^a	Species	Attenuated bacterial carrier ^b	Delivery	Systemic response ^c		Mucosal response		Protection	Reference
					Ab	CMI	Ab	CMI		
<i>Listeria monocytogenes</i>	ActA and LLO	Mouse	<i>S. typhimurium</i>	Oral	+	+	+	+	+	Darji <i>et al.</i> 1997, 2000
<i>Mycobacterium tuberculosis</i>	Ag85A, Ag85B, MPB/MPT51	Mouse	<i>L. monocytogenes</i>	Intraperitoneal intravenous	ND	+	ND	ND	+	Miki <i>et al.</i> , 2004
<i>Chlamydia trachomatis</i>	MOMP _e	Mouse	<i>S. typhimurium</i>	Oral	ND	ND	ND	ND	+	Brunham and Zhang, 1999
<i>Brucella abortus</i>	BFRf and P39	Mouse	<i>Y. enterocolitica</i>	Intragastric	+	+	ND	ND	+	Al-Mariri <i>et al.</i> 2002
<i>Clostridium tetani</i>	Tetanus toxin fragment C	Guinea pig	<i>S. flexneri</i>	Intranasal	+	ND	+	ND	+	Anderson <i>et al.</i> , 2000 Pasetti <i>et al.</i> , 1999
		Mouse	<i>S. typhi</i>	Intranasal	+	+	ND	+	ND	
<i>Helicobacter pylori</i>	HP-NAP	Mouse	<i>S. typhimurium</i>	Oral	+	ND	+	ND	ND	Sun <i>et al.</i> , 2006
<i>Mycoplasma hyopneumoniae</i>	P97R1	Mouse	<i>S. typhimurium</i>	Oral	-	+	-	ND	ND	Chen <i>et al.</i> , 2006a
	NrdF	Mouse	<i>S. typhimurium</i>	Oral	-	+	-	ND	ND	Chen <i>et al.</i> , 2006b

^a ActA: actin nucleator; LLO: Listeriolysin; Ag: antigen; MPB/MPT51: mycobacterial protein secreted by *M. bovis* /mycobacterial protein secreted by *M. tuberculosis*; MOMP: major outer membrane protein; BFRf: bacterioferritin; HP-NAP: *Helicobacter pylori* neutrophil activating protein; P97R1: P97 adhesin repeat region R1; NrdF: ribonucleotide reductase R2 subunit

^b *S. typhimurium*: *Salmonella typhimurium*; *L. monocytogenes*: *Listeria monocytogenes*; *Y. enterocolitica*: *Yersinia enterocolitica*; *S. flexneri*: *Shigella flexneri*; *S. typhi* : *Salmonella typhi*

^c ND: not determined, +: induced; -: not induced

from the cell (e.g. by apoptosis) and presented by bystander APC (Janeway and Medzhitov, 2002; Schoen *et al.*, 2004; Xu and Ulmer, 2003). Furthermore, an innate immune response could be generated that promotes the development of adaptive immune responses against the expressed antigens because a short-lived infection is established after delivery of these bacteria (Xu and Ulmer, 2003). This bacteria-mediated delivery of DNA vaccines to rodents has successfully been used for vaccination against a variety of infectious diseases of both viral (Table 7) and bacterial origin (Table 8). Immunizations were orally with *S. typhimurium* and intranasally with *Shigella* and a mucosal as well as a systemic immune response could be demonstrated.

1.6.3 Heterologous prime – boost immunization for enhanced mucosal immunity

In efforts to improve mucosal immune responses to naked DNA vaccines, heterologous prime – boost strategies combining parenteral DNA vaccination with mucosal delivery of live attenuated microorganisms or protein-based vaccines have also been proposed. Weeratna and colleagues (2000) reported the induction of antigen-specific IgA by parenteral priming of mice with DNA followed by a mucosal protein boost, whereas this was not observed with either route alone. In line with these results, parenteral priming with a DNA vaccine followed by oral boosting with a recombinant *Salmonella* strain, both encoding the structural subunit of ETEC CFA/I fimbriae, elicited systemic (serum IgG) and mucosal (fecal IgA) antibody responses (Lasaro *et al.*, 1999; 2004). In addition, dams vaccinated by the heterologous protocol passively protected their suckling pups against a lethal intragastric ETEC challenge, whereas this was not the case for dams vaccinated by either route alone (Lasaro *et al.*, 2005). These data suggest that, at least in mice, parenteral DNA vaccination followed by a heterologous mucosal boost is an effective strategy for the induction of mucosal immunity. However, less promising results were obtained when this strategy was applied to pigs (Yuan *et al.*, 2005). In pigs, IM priming with DNA followed by an oral boost with attenuated human rotavirus (AttHRV) failed to induce significant protection against virus shedding after rotavirus challenge (Yuan *et al.*, 2005). Conversely, significant protection against rotavirus shedding could be observed when the oral AttHRV vaccine was given to prime and the IM DNA vaccine to boost the immune system of pigs. The higher protection obtained by the oral AttHRV prime – IM DNA boost immunization was associated with higher mucosal IgA and IgG responses and significantly higher serum IgA titers (Yuan *et al.*, 2005). Their data indicate that the mucosal route is more effective than the parenteral route to prime a mucosal response and therefore might be more suitable to obtain protective immunity in large animals.

PART II

AIMS OF THE STUDY

Aims of the study

Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of diarrhoea in recently weaned piglets. To obtain protective immunity against ETEC, early priming of the intestinal mucosal immune system is necessary. At present no commercial vaccine allows successful vaccination. This is partly due to the presence of maternal antibodies hampering the induction of cellular and humoral immunity following conventional vaccination. Conversely, maternal antibodies won't interfere with the induction of cellular immunity by DNA vaccines, as these vaccines produce their encoded antigen endogenously. Hence, DNA vaccination could be an interesting approach to prime immunity against postweaning diarrhoea. However, parenteral immunization with our previous pcDNA1/faeG19 DNA vaccine encoding the fimbrial adhesin (FaeG) of F4⁺ ETEC only induced poor systemic immune responses in pigs.

The general aims of this study are to improve the immunogenicity of the FaeG DNA vaccine and to analyze whether parenteral DNA vaccination could reach the intestinal mucosal immune system.

For the optimization of the parenteral FaeG DNA vaccine, the following questions are addressed:

- What is the optimal route for parenteral FaeG DNA vaccination, the intradermal or the intramuscular route? (Chapter 2)
- Does plasmid encoded granulocyte-macrophage colony-stimulating factor (GM-CSF) pre-treatment enhance the immunogenicity of the FaeG DNA vaccine? (Chapter 2)
 - Can we demonstrate a chemotactic effect of GM-CSF DNA on professional antigen-presenting cells (APC)? (Chapter 3)
- Does optimization of the FaeG expression and/or co-administration of plasmid vectors encoding the A and B subunit of the thermolabile enterotoxin (LT) of *E. coli* (the LT vectors) enhance the immunogenicity of our parenteral DNA vaccine? (Chapter 4)

To analyze priming of the intestinal mucosal immune system, the following issues are investigated:

- Does priming with the FaeG DNA vaccine in the presence or absence of the LT vectors followed by an oral F4 protein boost induce intestinal mucosal antibody responses? (Chapter 4)
 - Does this protocol protect against challenge? (Chapter 4)

- Does co-administration of 1alpha,25-dihydroxyvitamin D3 (vitD3) to the optimized DNA vaccine further enhance this mucosal priming and/or further modulate the antibody response towards IgA? (Chapter 5)
- What is the importance of the oral F4 boost in the induction of systemic and/or mucosal immunity? (Chapter 5)
- If co-administration of the LT vectors and/or vitD3 enhance systemic and/or mucosal immunity, is this accompanied by an altered cytokine expression profile at the immune induction site? (Chapter 5)

PART III

**EXPERIMENTAL
STUDIES**

CHAPTER 2

Plasmid-encoded GM-CSF induces priming of the F4-specific serum IgA response by FaeG DNA vaccination in pigs

Based on: Melkebeek V, Verdonck F, Stuyven E, Goddeeris B, Cox E. (2006). Plasmid-encoded GM-CSF induces priming of the F4-specific serum IgA response by FaeG DNA vaccination in pigs. Vaccine 24 : 4592-4594.

ABSTRACT

We have used FaeG DNA to immunize piglets by the intradermal (ID) and the intramuscular (IM) route in a heterologous prime/boost model. ID immunization with DNA resulted in a better induction of cellular immunity, whereas only the IM immunization could prime an F4-specific serum IgA response. However, ID administration of plasmid-encoded GM-CSF one week before the ID immunization enhanced the F4-specific humoral and cellular immune response and even primed the F4-specific IgA response more efficiently than the IM immunization did.

INTRODUCTION

Newly weaned piglets are highly susceptible to F4⁺ enterotoxigenic *E. coli* (ETEC) induced diarrhea. To protect piglets against this infection, an F4-specific immune response should be induced during the suckling period, often in the presence of maternal antibodies. Since DNA vaccines are believed to be superior to protein vaccines in priming immune responses in young animals in the presence of maternal antibodies (Bot and Bona, 2002), priming with DNA during the suckling period combined with an F4 protein boost after weaning could be an interesting approach to obtain protection shortly after weaning. Hereto, an FaeG DNA vaccine (pWRGFaeG) encoding the adhesin of F4 fimbriae was constructed. In the present study, the capacity of the pWRGFaeG DNA vaccine was tested to prime B- and T-cell responses in F4-seronegative pigs by ID or IM injection. Hereby we also analysed the effect of the ID delivery of plasmid DNA encoding the porcine GM-CSF (pcDNA3-rpGM-CSF) (Inumaru and Takamatsu, 1995) on ID immunization.

METHODS

The sequence encoding the mature FaeG was obtained by PCR from *E. coli* strain GIS 26 (O149:F91:F4ac, LT⁺STa⁺STb⁺) and digested with NheI and BamHI (sites specified by the PCR primers) for insertion in the pWRG7079 vector, resulting in pWRGFaeG. The pWRG7079 vector contains the coding sequence for the human tissue plasminogen activator, allowing the extracellular secretion of proteins whose coding sequence is inserted in the correct reading frame at the NheI site. Expression and secretion of the FaeG was

demonstrated in COS-7 cells (data not shown). Subsequently, an immunization experiment was performed using 21 8-week-old conventional F4-seronegative pigs (Table 1). Seven days before the first immunization, 7 pigs were injected ID in the neck with 250 µg pcDNA3-rpGM-CSF (n=5) or the empty pcDNA3 vector (n=2). These pigs were immunized ID at the injection site with 500 µg pWRGFaeG (GM-CSF + ID group) or pWRG7079 (GM-CSF + ID controls) respectively on days 0 and 31. The remaining pigs were immunized both these days with 500 µg pWRGFaeG by the IM (n=5, IM group) or the ID route (n=5, ID group) or with 500 µg pWRG7079 by the ID (n=2, ID controls) or the IM route (n=2, IM controls). Four weeks after the second DNA immunization (day 59), all the pigs were intramuscularly boosted with 100 µg purified F4 fimbriae (Van der Stede *et al.*, 2002). F4-specific serum IgG and IgA responses were determined by an F4-specific ELISA and F4-specific cellular immune responses were analysed by a lymphocyte proliferation assay (Verdonck *et al.*, 2005). Differences between the groups in log₂ antibody titers and in lymphocyte proliferation (stimulation indexes, SI) were tested for statistical significance (P<0.05) using General Linear Model (Repeated Measure Analysis of Variance) with the Bonferroni adjustment for multiple comparisons and a Mann-Whitney U test, respectively.

Table 1: Experimental design

Group	n	D-7	D0 and D31	D59
IM	5	/	IM pWRGFaeG	IM F4
ID	5	/	ID pWRGFaeG	IM F4
GM-CSF+ ID	5	ID pGM-CSF	ID pWRGFaeG	IM F4
Control	2	/	IM pWRG7079	IM F4
	2	/	ID pWRG7079	IM F4
	2	ID pcDNA3	ID pWRG7079	IM F4

RESULTS

F4-specific serum antibody responses

After the second immunization with pWRGFaeG, a weak F4-specific serum IgG antibody response was seen in 3 out of 5 pigs of both the ID and the IM group and in all 5 pigs of the GM-CSF + ID group. No F4-specific IgG antibodies were detected in the control animals and none of the pigs showed an F4-specific IgA antibody response (Figure 1).

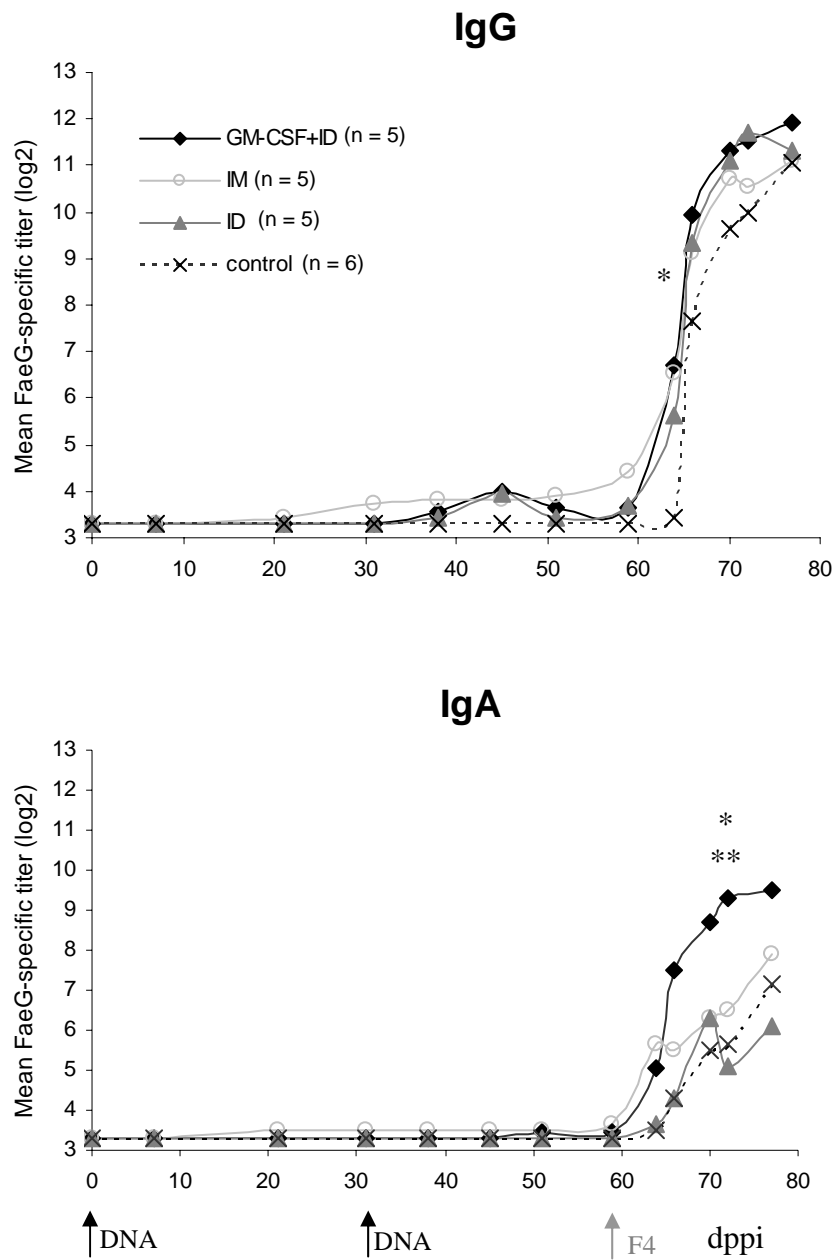


Figure 1. Kinetics of the F4-specific IgG and IgA serum response following intradermal injection of pcDNA3-rpGM-CSF (GM-CSF, day -7), 2 intramuscular (IM) or intradermal (ID) immunizations (days 0 and 31 post primary immunization (dppi), black arrows) with pWRGFaeG or pWRG7079 (controls) and an F4 protein boost (day 59, all pigs, grey arrow). Significant differences ($P < 0.05$) between the GM-CSF + ID group and the control group are indicated with * and between the GM-CSF + ID group and the ID group with **.

The F4 protein boost induced a secondary IgG response in the pWRGFaeG vaccinated groups, resulting in a 4.7-, 8.6- and 9.9-fold higher mean IgG titer in the ID, IM and GM-CSF

+ ID group ($P= 0.045$), respectively, compared to the control group where no F4-specific antibody response could be observed at this time. Two days later, the mean IgG titer remained highest in the GM-CSF + ID group and became comparable in the IM and the ID vaccinated groups. At this time, the mean IgG titer in the control group was increased and only remained 3-fold lower compared to the IM and the ID group and 4.8 fold lower compared to the GM-CSF + ID group. A secondary F4-specific serum IgA response was also observed in the GM-CSF + ID and the IM group with, 5 days after the F4 boost, a 1.6- and a 2-fold increase in mean serum antibody titer, respectively. The mean serum IgA titer further increased in the GM-CSF + ID group and was significantly higher compared to the control pigs ($P= 0.039$) and the ID group ($P= 0.021$) 2 weeks after the boost.

F4-specific lymphocyte proliferation

Although stimulation indexes (SI) were low 11 days after the second DNA immunization, a significantly higher mean SI could be observed in each pWRGFaeG vaccinated group (IM group: $P= 0.045$; ID group: $P= 0.028$; GM-CSF + ID group: $P= 0.011$) compared to the control group. Furthermore, although not significant, the GM-CSF + ID group showed a higher mean SI than the IM group ($P= 0.117$). Thirteen days after the F4 protein boost, SI were increased in all groups and only the ID group still had a significantly higher mean SI ($P= 0.028$) than the control group.

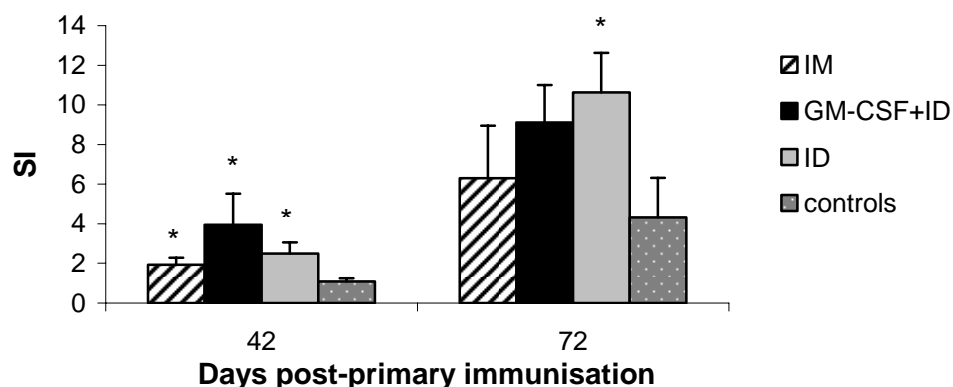


Figure 2. F4-specific proliferation of PBMC 11 days after the second immunization (day 42) and 13 days after the protein boost (day 72). Results are presented as means of the SI + SEM. Significant differences ($P < 0.05$) with the control group are indicated with *.

DISCUSSION

Our results have demonstrated that pWRGFaeG could efficiently prime F4-specific humoral and cellular immune responses in pigs. Moreover, results obtained with this plasmid were better than those observed with our previous FaeG DNA vaccine (Verfaillie *et al.*, 2004). In the absence of pcDNA3-rpGM-CSF, cellular immune responses were best induced after ID immunization, whereas only the IM immunization primed an IgA response. Injection of pcDNA3-rpGM-CSF 7 days before the first ID DNA vaccination improved F4-specific cellular and humoral immune responses and even primed a serum IgA response. The immune enhancing effect of plasmid-encoded GM-CSF is in line with previous studies (Bowne *et al.*, 1999; Somasundaram *et al.*, 1999) and may be due to a better transfection efficiency of APC since DNA encoded GM-CSF has been shown to recruit these cells to the injection site in mice (Bowne *et al.*, 1999). However, it remains to be proven whether this is also the case in pigs. Alternatively, GM-CSF expression might improve the functional capacities of DC (Curiel-Lewandrowski *et al.*, 1999).

To our knowledge, this is the first report demonstrating that GM-CSF is able to induce an IgA priming following intradermal immunization. Priming for a serum, and in addition a mucosal IgA response was also seen in mice with vitamin D or ADP-ribosylating toxins following intradermal protein immunization and was associated with an altered maturation and migration of antigen-pulsed DC resulting in their enhanced localization in the Peyer's patches (Enioutina *et al.*, 2000). Further research will be necessary to elucidate if a similar mechanism is responsible for the GM-CSF induced priming of IgA and thus if ID GM-CSF will result in a priming of the mucosal immune system. This priming is necessary to stimulate immunity against ETEC.

In conclusion, pWRGFaeG has the potential to prime F4-specific humoral and cellular immune responses in pigs following IM and ID immunization. A good priming of the FaeG-specific serum IgA response however, was only induced if pcDNA3-rpGM-CSF was injected ID 1 week before the first ID DNA vaccination. These results implicate that the combination of GM-CSF and ID DNA immunization is best suited to prime immune responses in vaccination trials against ETEC and other mucosal pathogens.

Acknowledgements

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Powderject Vaccines, Inc. and Pirbright Laboratory, IAH for providing pWRG7079 and pcDNA3-rpGM-CSF, respectively. We also wish to acknowledge Griet De Smet, Denise Slos and Rudy Cooman for their technical assistance.

CHAPTER 3

Effect of plasmid DNA encoding the porcine granulocyte-macrophage colony-stimulating factor on antigen- presenting cells in pigs

Melkebeek V, Van den Broeck W, Verdonck F, Goddeeris BM, Cox E. (2007) Effect of plasmid DNA encoding the porcine granulocyte-macrophage colony-stimulating factor on antigen-presenting cells in pigs. Vet. Immunol. Immunopathol. Accepted with moderate revision.

ABSTRACT

We previously demonstrated that intradermal (ID) delivery of plasmid DNA encoding the porcine granulocyte-macrophage colony-stimulating factor (GM-CSF) seven days before DNA vaccination enhances both cellular and humoral responses in pigs. In the present work, we studied the effect of the GM-CSF gene on antigen-presenting cells (APC) in pigs. We demonstrated that ID delivery of this gene significantly increased the number of epidermal CD1⁺ cells (Langerhans' cells, skin dendritic cells) at the injection site at day seven. This was accompanied by an enhanced percentage of APC at the immune induction site following DNA vaccination, whereas a positive effect on APC maturation could not be demonstrated. Taken together, our data suggest that both DC recruitment to the immunization site and expansion of APC in the draining LN following DNA vaccination might contribute to the immune enhancing effect of plasmid encoded GM-CSF in pigs.

INTRODUCTION

Professional antigen-presenting cells (APC) are critically involved in the induction of immunity following DNA vaccination by presenting antigen to T cells via MHC class I when directly transfected or through cross-presentation and via MHC class II when antigen is captured (Shedlock and Weiner, 2000). Furthermore, untransfected lymph node APC might also contribute to the immune response by production of cytokines induced by the DNA, so improving the conditions for T cell priming. Consequently, it is likely that adjuvants that expand the APC pool and/or enhance the potency of these cells would greatly improve the efficacy of DNA vaccines. In this regard, GM-CSF has received considerable attention given its role not only as a growth and differentiation factor for APC but also as a chemo-attractant for these cells (Kaplan *et al.*, 1992). Indeed, plasmid encoded GM-CSF has been shown to recruit APC to the injection site and expand the number of APC in the draining lymph node in mice (Bowne *et al.*, 2000; Haddad *et al.*, 2000). In pigs, plasmid encoded GM-CSF has been observed to enhance antibody responses (Andrew *et al.*, 2006; Cedillo-Baron *et al.*, 2001; Somasundaram *et al.*, 1999), Th1- as well as Th2-cell responses (Dufour *et al.*, 2000) and protection against viral challenge (Somasundaram *et al.*, 1999; Andrew *et al.*, 2006). While these studies co-injected the plasmid encoded GM-CSF with the DNA vaccine, several other studies suggest that the period between cytokine DNA injection and DNA vaccination might be critical for obtaining a maximal adjuvant effect (Kusakabe *et al.*, 2000; Barouch *et al.*,

1998). We have previously evaluated the adjuvant effect of plasmid encoded GM-CSF on ID DNA vaccination in pigs by injecting it 7 days before the first immunization, as plasmid encoded GM-CSF has been shown to attract APC to the injection site in mice with a maximal effect on day 7 (Bowne *et al.*, 2000). This protocol resulted in enhanced cellular and humoral responses and in priming of a serum IgA response in pigs (Melkebeek *et al.*, 2006). However, as results obtained in mice often do not translate to larger animals (Hengge *et al.*, 1996), further research on the mechanism of action of GM-CSF might be helpful to understand and fully exploit its immune enhancing effect in these species.

In the present study, we evaluated the influence of ID injection of GM-CSF DNA seven days prior to DNA vaccination on the density of epidermal APC at the immunization site and on the percentage of APC at the immune induction site following DNA vaccination of pigs.

MATERIALS AND METHODS

Plasmids

The pcDNA3-rpGM-CSF plasmid encoding the porcine GM-CSF was a gift of Pirbright Laboratories, IAH. The pWRGFaeGopt DNA vaccine was constructed as previously described (Melkebeek *et al.*, 2007). pcDNA3-rpGM-CSF, the empty pcDNA3.1 vector (Invitrogen, Leek, The Netherlands) and pWRGFaeGopt were propagated in *E. coli* DH5 α . Strains were grown in Luria Broth supplemented with ampicillin (100 μ g/ml) and plasmids were purified using the Endofree Plasmid Mega kit (Qiagen, Germany).

Monoclonal antibodies

The monoclonal antibodies (mAb) used are presented in Table 1.

Table 1. Monoclonal antibodies

Specificity	Clone	References	Distribution
CD1	76-7-4	Pescovitz <i>et al.</i> , 1984	cortical thymocytes, B cells, Langerhans cells, thymus DC, monocyte-derived DC, skin DC
MHCII	MSA3	Hammerberg and Schurig, 1986	Monocytes, macrophages, Langerhans cells, DC, B cells
SWC3a	74-22-15	Pescovitz <i>et al.</i> , 1984	Monocytes, macrophages, granulocytes, thymus DC, monocyte-derived DC, subpopulation of skin DC,

Experimental design

Fifteen conventional pigs (Belgian Landrace x Piétrain) were weaned at the age of 4 weeks. Subsequently, they were housed in isolation units where they obtained water and food ad libitum. At the age of 5 weeks, all pigs obtained at the left site of the neck an intradermal (ID) injection with 250 µg of pcDNA3-rpGM-CSF, whereas the right site was injected with 250 µg of the empty pcDNA3.1 vector (n=7, vector group), with sterile PBS (n=7, PBS group) or was not injected (n=1, control). Seven days later, at the same moment that immunization occurred in our previous study (Melkebeek *et al.*, 2006), the control pig, 1 pig of the vector group and 2 pigs of the PBS group were euthanized and punch biopsies with a diameter of 8 mm were taken at each injection site and also at an uninjected site of the neck to analyze the influence of GM-CSF on the density of APC at the immunization site. These biopsies were snap frozen in liquid-nitrogen-cooled isopropanol and stored at -70°C. At the same time, the remaining pigs were ID immunized with 500 µg of pWRGFaeGopt at the injection sites in the left and right site of the neck to analyze the effect of GM-CSF on DC recruitment to the immune induction site. Two and 4 days post immunization (pi), 2 PBS and 2 pcDNA3.1 controls and 7 days pi 1 PBS and 2 pcDNA3.1 controls were euthanized and the local draining cervical superficial dorsal lymph nodes (Cerv LN) were isolated to analyze the percentage of APC (Table 2). Euthanasia was performed by intravenous injection of pentobarbital (24 mg/kg; Nembutal®, Sanofi Santé Animale, Brussels, Belgium) followed by exsanguination.

Each ID injection was performed by multiple injections of plasmids diluted in a total volume of 1 ml sterile PBS.

Table 2. Experimental design

Group	n	D-7		Euthanasia	D0 Immunization (left + right)	D2 Euthanasia	D4 Euthanasia	D7
		left injection	right injection					
control	1	pGM-CSF	/	1	/	/	/	/
PBS control	7	pGM-CSF	PBS	2	pWRGFaeGopt (n=5)	2	2	1
Vector control	7	pGM-CSF	pcDNA3	1	pWRGFaeGopt (n=6)	2	2	2

Quantification of APC cells in porcine epidermis

To quantify the numbers of APC in the epidermis, the CD1⁺ cells (Table 1) were counted since CD1 expression in this tissue is restricted to Langerhans' cells and skin DC. Hereto,

transversal 5 μm thick cryostat sections from the skin biopsies were fixed in acetone at -20°C for 10 min, air dried and washed in PBS. Subsequently, sections were sequentially incubated in a humidity chamber at 37°C with 10% sheep serum for 30 min to block non-specific binding sites, with a CD1-specific mAb for 1 h to identify APC and with a sheep anti-mouse IgG (whole molecule) Fab-fragment labelled with FITC (Sigma, Bornem, Belgium) for 1 h. After each step, the sections were washed 3 times with PBS. Finally, they were air-dried and mounted in a glycerin solution containing DABCO. Quantification of CD1⁺ cells was performed on digital micrographs using the AnalySIS 3.2 software. On each micrograph, the epidermal basal membrane was marked polygonally and epidermal CD1⁺ cells were counted and expressed per mm basal membrane. Only CD1⁺ cells with clearly visible cell bodies were counted. For each biopsy, a minimum of 5 sections (at least 50 μm apart) with a total basal membrane length of at least 20 mm were analyzed (Figure 1).

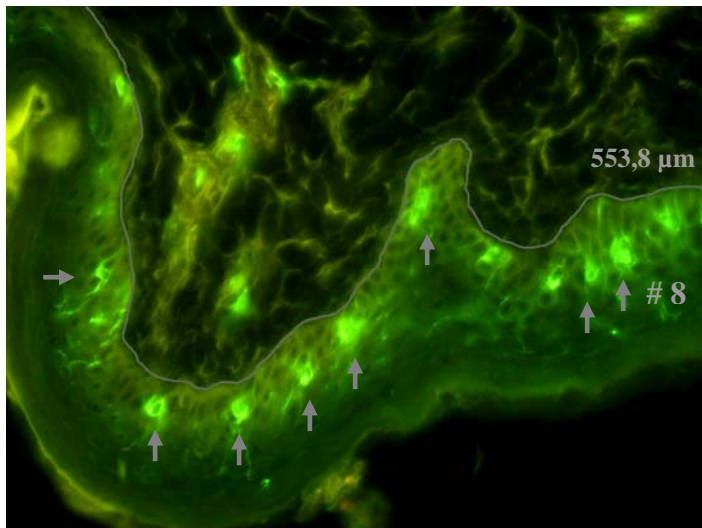


Figure 1. CD1-positive cells in the epidermis at day 7 after injection of PBS. The epidermal basal membrane was polygonally marked and fluorescent cells with a clearly visible cell body were counted.

Isolation of cervical superficial dorsal lymph node monomorphonuclear cells

Monomorphonuclear cells (MC) were isolated from the draining Cerv LN as described by Vremec and Shortman (1997) with minor modifications. Cerv LN were cut into small pieces in medium [RPMI-1640 (GIBCO BRL, Paisley, Scotland) + 20 mM HEPES (GIBCO BRL) + 2% FCS + 100 IU/ml penicillin (GIBCO BRL) + 100 $\mu\text{g}/\text{ml}$ streptomycin (GIBCO BRL) + 0.01% (wt/vol) DNase I (Boehringer Mannheim, Brussels, Belgium)] to which 0.005% (wt/vol) collagenase (SERVA, Polylab, Antwerp, Belgium) was added. After vigorous pipetting, the medium, containing cells in suspension, was collected. The remaining tissue

pieces were further digested in this medium supplemented with 0.1% (wt/vol) collagenase for 25 min at room temperature while gently shaking. Then, EDTA (pH 7.2) was added to a final concentration of 10 mM to disrupt DC-T cell complexes and incubation was continued for 5 min. Next, undigested tissue was removed by passage through a cell strainer whereafter cells in suspension were pelleted by centrifugation (380g at 18 °C for 10 min) and resuspended in washing buffer [equal volumes of PBS and Alsever's solution + 100 IU/ml penicillin + 100 µg/ml streptomycin + 1% FCS + 0.1 mM EDTA].

MC were isolated by density gradient centrifugation (500g at 18 °C for 45 min) on Lymphoprep® (Invitrogen, Merelbeke, Belgium). Finally, the MC were washed with washing buffer and resuspended in ice-cold staining buffer (PBS + 2% sheep serum + 0.2 mM EDTA) at a final concentration of 10^7 cells/ml.

Flow cytometric analysis

MC were analysed for expression of cell surface antigens by flow cytometry. Hereto, cells (10^6 cells/ well) were incubated for 30 min on ice in staining buffer containing a swine CD1-, MHC class II (MHCII)- or SWC3-specific mAb (Table 1) and subsequently in staining buffer containing the FITC-labelled sheep anti-mouse IgG. Staining buffer without Ab was used as a negative control. Incubations occurred for 30 min on ice after which the cells were washed 3 times in staining buffer. Finally, cells were resuspended in 600 µl staining buffer supplemented with 6µl propidiumiodide (1 µg/ml) to exclude death cells during analysis. Flow cytometric analysis was performed on a FACSCanto flow cytometer (Becton Dickinson Immunocytometry systems) and at least 10,000 viable cells were analysed per run.

Statistical analysis

Data were tested for normal distribution with a Shapiro-Wilk test. Statistical analysis was done using a paired-sample T-test (SPSS 12.0 for windows). $P < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

pcDNA3-rpGM-CSF increases APC numbers in the epidermis

Seven days after ID injection of plasmid encoded GM-CSF, an increase in epidermal CD1-positive cells was observed in all four examined pigs (mean increase of 36 % compared to uninjected epidermis; $P = 0.004$). This effect was due to the expression of GM-CSF rather than

to the presence of CpG motifs in the vector or to inflammation caused by the injection on itself as no clear increase could be observed following injection of the empty pcDNA3.1 vector or PBS, respectively (Table 3).

These data are in line with studies in mice demonstrating APC recruitment by plasmid encoded GM-CSF (Bowne *et al.*, 1999; Haddad *et al.*, 2000), Flt3-ligand and/or MIP1 α (McKay *et al.*, 2004; Sumida *et al.*, 2004). In these studies, APC recruitment was consistently accompanied by an enhanced vaccine induced immune response. Moreover, the Sumida study demonstrated that blocking DC recruitment, by disrupting the chemokine gradient with a high-dose of MIP-1 α protein or by delivering the chemokine plasmids at a site different from the DNA vaccine, abrogated the adjuvanticity of the plasmid encoded cytokines, confirming the importance of locally recruited APC in mice. Likewise, the increased number of CD1⁺ cells in the present study suggests that APC recruitment to the immunization site has also contributed to the immune enhancing effect of plasmid GM-CSF in pigs (Melkebeek *et al.*, 2006). Possible mechanisms for an enhanced immunogenicity by APC recruitment include a higher transfection efficiency and/or an enhanced uptake of plasmid encoded antigen by these cells.

Table 3: Number of CD1⁺ - cells per mm epidermal basal membrane at the immunization site

Group	GM-CSF injection site	Control site		
		Uninjected	PBS	pcDNA3
control	18.9	15,3	/	/
PBS control	17.9	12,4	14.3	/
PBS control	18.9	14,0	14.6	/
Vector control	22.3	15,5	/	14.3
Mean \pm SEM	19.5 \pm 1.0*	14.3 \pm 0.7	14.4 \pm 0.2	14.3

* : significant difference between the pcDNA3-rpGM-CSF and the uninjected site

pcDNA3-rpGM-CSF injection increases the percentage of APC in the local draining Cerv LN

To evaluate the influence of GM-CSF pre-treatment on the percentage of APC at the immune induction site following DNA vaccination, we analysed the percentage of MHCII⁺, CD1⁺ and SWC3⁺ cells in the Cerv LN at days 2, 4 and 7 pi with pWRGFaeGopt. At day 2 pi, the percentage of CD1⁺, SWC3⁺ and MHCII⁺ cells was 2.3- to 2.7-fold increased at the pcDNA3-rpGM-CSF injection site compared to the PBS pre-treatment site (n=2), whereas no increase for MHCII and only a 1.4- to 1.8- fold increase for CD1 and SWC3 was observed

compared to the pcDNA3.1 pre-treatment site (n=2) (Figure 2). The increases in the percentage of CD1⁺ (P=0.042) and SWC3⁺ cells (P=0.021) were only significant when the PBS and the vector pre-treated site were considered together (n=4; further referred to as the control site) (Figure 2). Later on, the positive effect of plasmid GM-CSF pre-treatment steadily disappeared. Indeed, at day 4 pi, GM-CSF pre-treatment only significantly increased the percentage of SWC3⁺ cells (P=0.035) and at day 7 pi, no significant effects were observed anymore. However, it can not be excluded that a better T cell proliferation at the GM-CSF pre-treated site, resulting in a higher number of T cells, masked the effect of GM-CSF on APC recruitment at these later time points. Indeed, an increased lymphocyte proliferation has been observed after DNA vaccination of pigs which were pre-injected with the GM-CSF plasmid (Melkebeek *et al.*, 2006).

As surface expression of MHCII becomes increased several fold during APC maturation (Villadangos *et al.*, 2005), surface MHCII expression was used to evaluate APC maturation. A positive effect of GM-CSF on APC maturation could not be demonstrated at any of the examined time points as surface expression of MHCII on lymph node APC was similar at the GM-CSF and the PBS or vector pre-treated site (data not shown).

Our present results are in agreement with observations in mice demonstrating a positive effect of GM-CSF pre-treatment on APC recruitment into the draining LN 24 h after gold particle bombardment (Bowne *et al.*, 1999). Moreover, Curiel-Lewandrowski *et al* (1999) observed a correlation between the enhanced migratory capacity of DC and their enhanced capacity to induce primary responses following GM-CSF gene transfection. Taken together, their and our data suggest that both DC recruitment to the immunization site and expansion of APC in the draining LN following DNA vaccination might contribute to the enhanced immunogenicity observed following plasmid GM-CSF pre-treatment. However, a possible contribution of other factors independent of APC to the immune enhancing effect of plasmid GM-CSF, such as extension of the life span of germinal centers favoring the interaction between follicular DC and B cells, a direct effect on follicular DC or an enhanced avidity of the interactions between B- and T-cells (Gerloni *et al.*, 1998) was not addressed in the present study.

Acknowledgements

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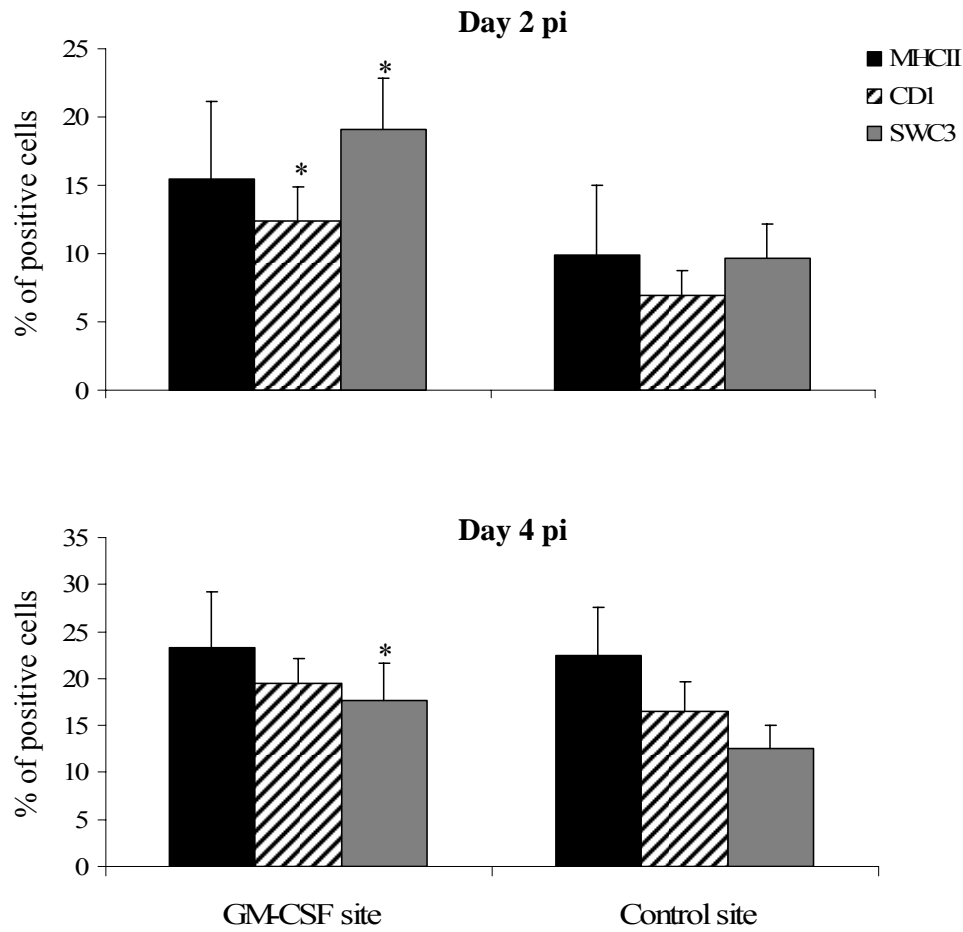


Figure 2. GM-CSF DNA increases the percentage of APC in the local draining Cerv LN following DNA vaccination. Pigs were ID injected with GM-CSF DNA at the left site (GM-CSF site) and with the empty vector or PBS at the right site of the neck (collectively referred to as the control site). Seven days later, they were immunized at both sites with pWRGFaeGopt and at day 2, 4 and 7 pi, the percentage of CD1⁺, SWC3⁺ and MHCII⁺ cells was determined in the Cerv LN. Results are presented as means \pm standard deviation.

CHAPTER 4

Optimized FaeG expression and LT DNA adjuvant enhance priming of an intestinal immune response by an FaeG DNA vaccine in pigs

Melkebeek V, Sonck E, Verdonck F, Goddeeris BM, Cox E. (2007) Optimized FaeG expression and LT DNA adjuvant enhance priming of an intestinal immune response by an FaeG DNA vaccine in pigs. Clin Vacc Immunol 14:28-35.

ABSTRACT

One of the problems hindering the development of DNA vaccines is the relatively low immunogenicity often seen in animals and humans compared to that in mice. In the present study, we tried to enhance the immunogenicity of a pcDNA1/*faeG*19 DNA vaccine in pigs by optimizing the FaeG expression plasmid and by co-administration of plasmid vectors encoding the A and B subunit of the *E. coli* thermo-labile enterotoxin (LT). Insertion of a Kozak sequence and optimization of vector- (cellular localization, expression) and both vector- and codon-usage were all shown to enhance the *in vitro* FaeG expression compared to pcDNA1/*faeG*19. Subsequently, pcDNA1/*faeG*19 and the vector- and vector- + codon-optimized construct were tested for their immunogenicity in pigs. In line with the *in vitro* results, antibody responses were better induced with increasing expression. The LT vectors additionally enhanced the antibody response, although not significantly, and were necessary to induce an F4-specific cellular response. These vectors were also added since LT has been described to direct the systemic response towards a mucosal IgA response in mice. Here however, the intradermal FaeG DNA prime-oral F4 boost immunization mainly resulted in a systemic IgG response, with only a marginal but significant reduction in F4⁺ *E. coli* faecal excretion when the piglets were primed with pWRGFaeGopt and pWRGFaeGopt with the LT vectors.

INTRODUCTION

Intestinal infections with enterotoxigenic *Escherichia coli* (ETEC) remain problematic for humans, pigs and calves. The bacteria possess adhesins which allow them to colonize the small intestine and to produce enterotoxins which act locally on enterocytes. These adhesins bind to specific receptors on the enterocyte brush borders, and absence of these receptors renders the animal resistant to bacterial colonization and consequently to ETEC induced diarrhea (Rutter *et al.*, 1975). In pigs, F4 (K88) fimbriae are the best characterised adhesins (Van den Broeck *et al.*, 2000). They are long proteinaceous appendages mainly composed of several hundreds identical adhesive subunits, called FaeG (Mol and Oudega, 1996).

Our laboratory developed a challenge model in pigs in which new vaccination strategies against ETEC can be tested. In this model, it was demonstrated that oral administration of F4 fimbriae to weaned F4R⁺ pigs led to protection against a subsequent F4⁺ ETEC infection (Van den Broeck *et al.*, 1999a). In practice, a protective mucosal immune response often needs to be induced in the presence of maternal antibodies.

Several studies have shown that DNA vaccines, in contrast to conventional vaccines, can successfully prime immune responses in the presence of maternal antibodies, pointing out its potential use in young animals (Bot and Bona, 2002). Our previous pcDNA1/*faeG*19 DNA vaccine however, was only marginally immunogenic in pigs (Verfaillie *et al.*, 2004). This is in agreement with other studies in large animals and man suggesting poor immunogenicity of DNA vaccines in these species (MacGregor *et al.*, 1998; van Rooij *et al.*, 1998). Several approaches have been explored to enhance the potency of DNA vaccines. These include optimization of expression (Montgomery *et al.*, 1993; Steinberg *et al.*, 2005), optimization of transfection efficiency (Babiuk *et al.*, 2003b), targeting of DNA or the encoded antigen to dendritic cells (Drew *et al.*, 2001; Singh *et al.*, 2000) and co-administration of adjuvants such as vector-encoded cytokines (Kim *et al.*, 2001; Melkebeek *et al.*, 2006). Promising results were also observed when DNA vaccines were used in heterologous prime-boost models (Guo *et al.*, 2004; Toussaint *et al.*, 2005).

In the present study, we evaluated the impact of a Kozak sequence, optimization of vector-usage (intron A, BGH polyA, tPA leader sequence) and codon-usage on the *in vitro* FaeG expression using 2 porcine, a human and a non-human primate cell line. Subsequently, the influence of expression optimization and of co-administration of plasmids encoding the LTA and LTB subunits (collectively named the LT vectors) was analyzed on the immunogenicity of the FaeG DNA vaccine in pigs. Additionally, priming of an F4-specific intestinal mucosal immune response was assessed in an intradermal (ID) DNA prime - oral F4 boost protocol.

MATERIALS AND METHODS

Isolation of F4 fimbriae

F4 fimbriae were isolated from ETEC strains GIS26 (O149:K91:F4ac, LT⁺STa⁺STb⁺) and IMM 01 (O147:F4ac, LT⁺STb⁺) as previously described (Van den Broeck *et al.*, 1999a). GIS26 F4 fimbriae were used in ELISA, ELISPOT and for oral immunizations. IMM01 fimbrial solutions were used in the FaeG-specific lymphocyte proliferation assay as they don't contain flagellin (Verdonck *et al.*, 2004).

Plasmids

pcDNA1/*faeG*19 and pWRGFaeG were constructed as previously described by cloning the GIS26 *faeG* sequence into pcDNA1 and pWRG7079, respectively (Verfaillie *et al.*, 2004; Melkebeek *et al.*, 2006). The latter vector contains the tPA signal sequence which allows

extracellular secretion of the vector encoded protein. The *faeGK* fragment was derived from pcDNA1/*faeG19* by PCR using a forward primer inserting a Kozak sequence (GCC ACC ATG G) around the ATG translation initiation codon (underlined) and was subsequently cloned into pcDNA1, resulting in pcDNA1/*faeGK*. The *faeG* sequence was optimized for porcine expression using Genscript's algorithm, increasing the codon-adaptation index (Sharp and Li, 1987) of the *faeG* from 0.542 to 0.811. This codon-optimized sequence was constructed synthetically (Genscript corp., Edison, USA) and subsequently cloned into pWRG7079, resulting in pWRGFaeGopt. The pcDNA3-rpGM-CSF plasmid encodes the porcine GM-CSF. pJV2004 and pJV2005 (Arrington *et al.*, 2002) encode the LTA and LTB subunit, respectively, behind a tPA signal sequence. Each construct was verified by sequencing the insert and its junction site and large-scale purification was conducted by Qiagen Endofree plasmid kits (Qiagen GmbH, Germany).

***In vitro* expression of recombinant FaeG**

Swine kidney (SK-6 and PK-15), human embryonic kidney (293T) and monkey kidney (COS-7) cells were grown in DMEM (GIBCO BRL, Paisley, Scotland) supplemented with penicillin (100 IU/ml) (GIBCO BRL), streptomycin (100 µg/ml) (GIBCO BRL), L-glutamine (400 mM) (GIBCO BRL) and 10% FCS. The cells (2×10^5 cells for each transfection) were transfected transiently using LipofectamineTM 2000 (Invitrogen, Merelbeke, Belgium) following the manufacturer's protocol. Twenty-four, 48, 72 and 96 hours after transfection, culture media (supernatants) were collected and centrifugated to remove cell debris, after which transfected cells were collected, resuspended in PBS and lysed by 3 times freezing (-80 °C) and thawing. Each transfection was done 4 times. The amount of FaeG was quantified by an F4-specific ELISA as described (Verfaillie *et al.*, 2004) using a serial dilution of a known concentration of purified F4 to establish the standard curve.

Immunization experiment

Thirty-three F4-seronegative conventionally bred pigs (Belgian Landrace x Piétrain) were weaned at the age of 4 weeks and housed in isolation units. Starting 1 day before weaning, all animals were orally treated during 5 successive days with colistine (150,000 U/kg body weight; Colivet, Prodivet Pharmaceuticals, Eynatten, Belgium) to prevent *E. coli* infections during the weaning period.

The experimental setup is shown in table 1. All pigs were injected ID at the left site of the neck with 250 µg of pcDNA3-rpGM-CSF at the age of 6 weeks. Seven and 28 days later

(days 0 and 21 post primary immunization (ppi)), the pigs were ID immunized at the pcDNA3-rpGM-CSF injection site with 500 µg of either pcDNA1/*faeG19* (n = 7, pcDNA1/*faeG19* group), pWRGFaeG (n = 7, pWRGFaeG group), pWRGFaeGopt (n = 7, pWRGFaeGopt group), pWRGFaeGopt supplemented with 100 µg pJV2004 and 100 µg pJV2005 (n = 6, pWRGFaeGopt + LT group) or the empty pWRG7079 vector (n = 6, control group). Plasmids were diluted in sterile PBS and given by multiple injections of a total volume of 1 ml following sedation of the pigs with azaperone (2mg/kg; Stressnil®, Janssen-Cilag, Beerse, Belgium). All the pigs were orally boosted with 1 mg F4 fimbriae at days 43, 44 and 45 ppi as described earlier (Van den Broeck *et al.*, 1999b). Two pigs of the control group and 3 pigs of each vaccinated group were euthanized at day 49 or 50 ppi to evaluate antibody responses in the spleen, mesenteric lymph nodes (Mes LN) (jejunal and ileal), jejunal Peyer's patches (JPP), ileal Peyer's patch (IPP) and lamina propria (LP) (Figure 3). The remaining pigs were challenged intragastrically with 10¹⁰ F4⁺ ETEC at days 51 and 52, respectively, as described by Cox *et al.* (1991). Three weeks after challenge, these pigs were euthanized. Euthanasia was performed by intravenous injection of pentobarbital (24 mg/kg; Nembutal®, Sanofi Santé Animale, Brussels, Belgium) followed by exsanguination. The experimental procedure was performed following the guidelines of the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University (EC2004/85).

Table 1. Experimental design

Group	N	Day -7	Days 0 and 21	Days 43, 44, 45	Localization of ASC at day 49 or 50	ETEC challenge at day 51-52
					N	N
pcDNA1/ <i>faeG19</i>	7	All groups : pcDNA3-rpGM-CSF	pcDNA1/ <i>faeG19</i>	All groups : Oral F4 protein	3	4
pWRGFaeG	7		pWRGFaeG		3	4
pWRGFaeGopt	7		pWRGFaeGopt		3	4
pWRGFaeGopt + LT	6		pWRGFaeGopt + pJV2004 + pJV2005		3	3
Control	6		pWRG7079		2	4

Samples and tests

Sera were sampled at regular intervals (Figure 1) and were used to determine the F4-specific IgG, IgA and IgM titer by an indirect ELISA (Van den Broeck *et al.*, 1999b). The cut off values in this ELISA were 0.82, 0.57 and 0.29 for IgM, IgG and IgA, respectively.

At day 35 ppi, peripheral blood MC (PBMC) were isolated (Van den Broeck *et al.*, 1999b) to determine the FaeG-specific lymphocyte proliferation (Van der Stede *et al.*, 2003).

At day 49 or 50 ppi, MC were isolated from the spleen, Mes LN (jejunal and ileal), JPP, IPP and LP to enumerate the number of IgG, IgA and IgM ASC per 5×10^6 MC (Van den Broeck *et al.*, 1999b). Furthermore, contents were collected from the duodenum, jejunum and ileum (Verdonck *et al.*, 2002) and were tested for FaeG-specific IgG and IgA by an indirect ELISA (Van den Broeck *et al.*, 1999b). Cut off values were 0.22 and 0.26 for IgG and IgA, respectively.

After challenge infection, faecal samples were collected daily till day 7 post challenge and the F4⁺ *E. coli* in these samples were enumerated by dot blotting as described (Van den Broeck *et al.*, 1999a).

The F4-receptor (F4R) status of all pigs was determined by an *in vitro* villus adhesion assay on small intestinal villi (Van den Broeck *et al.*, 1999c).

Statistical analysis

Statistical analysis was done using SPSS 12.0 for Windows. Differences in FaeG expression between different constructs within each cell line were tested with a paired-sample t test. Differences in log₂ serum antibody titers and in log₁₀ faecal ETEC excretions between groups were analyzed using General Linear Model (Repeated Measures Analysis of Variance) with the Bonferroni adjustment for multiple comparisons. Differences in log₂ serum antibody titers between different time points within groups were tested with a paired-sample t test. Differences in duration of faecal F4⁺ *E. coli* excretion between groups was tested using one-way ANOVA. Differences between groups in lymphocyte proliferation were analyzed with the Kruskal-Wallis test. P<0.05 was considered as statistically significant.

RESULTS

In vitro FaeG expression

Overall, insertion of a Kozak sequence in pcDNA1/*faeG*19 significantly increased the FaeG expression. As can be seen in Table 2, pcDNA1/*faeG*K induced a 1.6- to 4.5-fold increase in expression. Usage of pWRG7079 instead of pcDNA1 further enhanced this expression in 293T and COS-7 cells. However, only in COS-7 cells, this increase was significant compared to the expression by pcDNA1/*faeG*K. Expression in both porcine cell lines was not improved by pWRG7079. In fact, expression became similar again to the expression induced by pcDNA1/*faeG*19. Codon-optimization of FaeG in pWRGFaeG resulted in the highest expression in COS-7 as well as in 293T cells, whereas expression in SK-6 and

Table 2: Influence of expression optimization on the *in vitro* FaeG expression in COS-7, 293T, SK-6 and PK-15 cells

Cell line	Constructs	Total FaeG expression (ng/ 2x10 ⁵ cells) at ^a				FaeG in SN (% of total FaeG expression) at:				Optimized expression / unoptimized expression at ^b			
		24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h
COS-7	pcDNA1/faeG19	1.6 ± 0.6	3.8±0.5	5.8±1.1	6.8±1.4	0.0±0.0	61.2±13.5	61.9±7.8	89.4±8.1				
	pcDNA1/faeGK	7.7±2.2 (1)	7.9±1.1 (1)	14.2±3.1 (1)	12.7±3.3	0.0±0.0	29.5±8.3	55.0±8.5	93.4±2.1	4.5±0.1	2.1±0.3	2.5±0.2	2.4±0.6
	pWRGFaeG	21.0±3.5 (1,2)	36.7±4.8 (1,2)	47.1±6.8 (1,2)	52.3±9.1 (1)	18.4±8.7	31.7±8.5	50.6±9.2	67.3±7.3	9.4±1.4	10.0±1.5	8.5±1.0	8.3±1.9
	pWRGFaeGopt	22.4±1.4 (1,2)	41.8±1.4 (1,2)	55.2±4.8 (1,2)	54.5±11.6 (2,3)	16.1±7.5	31.2±4.7	56.2±8.1	76.8±6.9	11.2±1.3	11.5±1.1	10.4±1.6	10.2±2.3
293T	pcDNA1/faeG19	6.8±2.3	33.0±10.9	57.5±16.1	50.5±10.1	0.0±0.0	9.0±5.3	3.1±1.0	8.3±3.0				
	pcDNA1/faeGK	17.8±4.0 (1)	60.9±11.5	94.9±20.5 (1)	93.6±15.7 (1)	0.0±0.0	5.2±1.8	4.4±1.8	7.5±0.2	2.6±0.6	1.7±0.2	1.8±0.2	1.9±0.3
	pWRGFaeG	38.5±8.7 (1)	86.0±12.1 (1)	98.3±20.7	112.6±21.5 (1)	11.5±2.0	30.4±11.0	31.4±9.2	32.8±2.0	5.7±0.5	2.5±0.4	1.9±0.3	2.3±0.3
	pWRGFaeGopt	71.5±14.4 (1,3)	137.6±10.6 (1,2,3)	166.6±28.7 (1,2,3)	157.4±26.5 (1,2)	7.5±2.5	27.8±10.8	17.4±2.2	33.3±3.2	9.5±0.6	4.3±1.0	3.6±0.9	3.2±0.4
SK-6	pcDNA1/faeG19	14.7±5.4	21.5±2.7	29.8±0.5	32.4±8.1	0.0±0.0	15.5±5.1	38.4±5.9	56.7±5.8				
	pcDNA1/faeGK	27.4±8.1 (1)	43.2±3.3 (1,3)	47.2±2.9 (1,3)	49.1±10.8 (1)	0.9±0.9	15.2±3.0	43.1±6.0	68.5±7.0	2.1±0.3	2.1±0.2	1.6±0.1	1.6±0.1
	pWRGFaeG	16.1±4.1	15.9±4.5	26.5±6.9	27.0±8.7	13.2±10.3	40.9±9.8	76.9±3.1	84.6±5.7	1.2±0.1	0.9±0.3	0.9±0.2	0.9±0.2
	pWRGFaeGopt	35.6±8.1 (1,3)	37.7±8.0	41.2±9.7 (3)	42.1±7.8 (3)	13.6±8.5	44.1±5.2	72.1±2.7	84.9±4.6	2.7±0.3	1.9±0.6	1.4±0.3	1.5±0.4
PK-15	pcDNA1/faeG19	11.7±2.8	22.0±7.0	24.9±6.2	30.1±9.1	1.1±1.1	16.4±3.4	26.8±3.6	43.9±3.5				
	pcDNA1/faeGK	22.5±7.8	37.6±10.5 (1)	44.5±16.0	54.7±18.9	0.4±0.4	13.0±2.1	30.8±4.0	54.9±4.3	1.7±0.3	2.0±0.4	1.6±0.3	1.6±0.3
	pWRGFaeG	19.2±4.0 (1)	17.0±1.9	21.9±2.7	24.1±7.0	9.5±7.0	36.9±8.7	65.0±4.4	81.7±7.1	1.8±0.2	1.4±0.7	1.0±0.2	0.9±0.1
	pWRGFaeGopt	41.1±10.2 (1,2,3)	39.1±7.7 (1,3)	43.0±9.8 (1)	50.3±15.0 (1,3)	17.2±7.0	43.6±5.6	60.9±5.7	76.7±9.2	3.6±0.2	2.5±0.8	1.8±0.2	1.7±0.1

^a The calculated total includes FaeG expressed in cell lysates and FaeG expressed in supernatant (SN). The numbers 1, 2, and 3 in parentheses indicate significant increases ($P < 0.05$) compared to pcDNA1/faeG19, pcDNA1/faeGK, and pWRGFaeG, respectively.

^b Values are ratios. Optimized expression is the total amount of FaeG expressed after transfection with pcDNA1/faeGK, pWRGFaeG, or pWRGFaeGopt. Unoptimized expression is the total amount of FaeG detected after transfection with pcDNA1/faeG19. Results are the means of 4 transfections ± SEM.

PK-15 cells increased again towards the level of pcDNA1/*faeGK*. Only 24 h after transfection of the porcine cell lines, expression was higher. Taken together, vector-optimization improved the expression up to 1.2-, 1.8-, 5.7-, 10.0-fold and both vector- and codon-optimization increased the expression up to 2.7-, 3.6-, 9.5-, 11.5-fold in SK-6, PK-15, 293T and COS-7 cells, respectively, in comparison with pcDNA1/*faeG19* (Table 2).

Following transfection with the secretion constructs pWRGFaeG and pWRGFaeGopt, no correlation was seen between the total amount of FaeG expressed and the amount secreted in the supernatant. The fraction of FaeG in supernatant increased with time, whereas this increase was less pronounced for the total FaeG expression, but remained remarkably lower for 293T cells. Transfection with the non-secretion constructs pcDNA1/*faeG19* and pcDNA1/*faeGK* hardly resulted in FaeG in the supernatant after 24 h, but from then on, the percentage of FaeG in the supernatant also increased with time.

***In vivo* immunogenicity of the FaeG DNA vaccines and adjuvant effect of the LT vectors**

All pigs were ID injected with pcDNA3-rpGM-CSF 1 week before the first DNA vaccination as we previously reported that this injection enhanced the induction of F4-specific immune responses (Melkebeek *et al.*, 2006). To evaluate the influence of expression optimization on the immunogenicity of the FaeG DNA vaccine in pigs, we opted for pcDNA1/*faeG19* and pWRGFaeG because we already used these constructs in an immunization experiment (Verfaillie *et al.*, 2004; Melkebeek *et al.*, 2006) and for pWRGFaeGopt because it gave the highest FaeG expression in porcine cells. In addition, we evaluated the influence of the LT vectors as LT has previously been shown to induce a mucosal immune response when applied to the skin (Enioutina *et al.*, 2000; Glenn *et al.*, 2000).

F4-specific immune responses following intradermal DNA vaccination

ID DNA vaccination did not induce an F4-specific serum IgM response, whereas a statistically significant F4-specific serum IgA response was only detected in the pWRGFaeGopt group, from 2 weeks after the second immunization onwards. Nevertheless, most groups showed an IgG response of which the height and speed of onset increased in the order pcDNA1/*faeG19*, pWRGFaeG, pWRGFaeGopt (Figure 1). Indeed, none of the pigs of the pcDNA1/*faeG19* and the pWRG7079 group showed F4-specific serum IgG following DNA vaccination, whereas in the pWRGFaeG group, F4-specific IgG was observed in 1 pig on day 14 ppi and in 6 on 7 pigs 3 weeks after the second immunization. Even more pigs

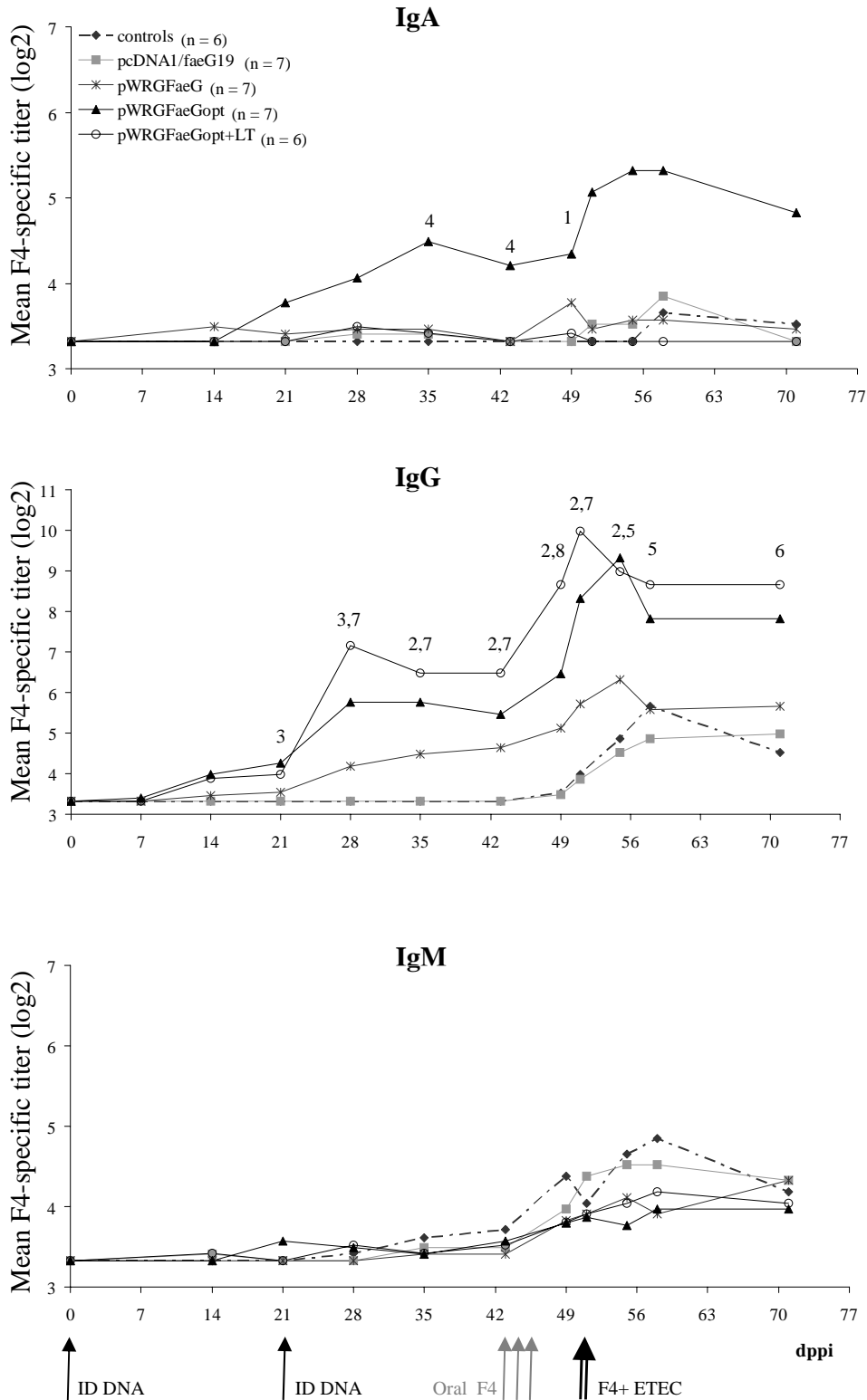


Figure 1: Mean F4-specific serum IgA, IgG and IgM titers following 2 ID immunizations (black arrows) with pcDNA1/*faeG*19, pWRGFaeG, pWRGFaeGopt, pWRGFaeGopt plus the LT vectors or pWRG7079 (controls), an oral F4 protein boost (all pigs, gray arrows) and an F4+ ETEC challenge (double arrow). Shown are significant differences ($P < 0.05$) of the pWRGFaeGopt group with (1) the pcDNA1/*faeG*19 group, (2) the control and the pcDNA1/*faeG*19 group, (3) the control, pcDNA1/*faeG*19 and the pWRGFaeG group, (4) all other groups, and of the pWRGFaeGopt + LT group with (5) the pcDNA1/*faeG*19 group, (6) the control and the pcDNA1/*faeG*19 group, (7) the control, pcDNA1/*faeG*19 and the pWRGFaeG group and (8) all other groups. Significant differences between time points are mentioned in the text.

showed F4-specific IgG in the pWRFaeGopt group, namely 4 on 7 pigs on day 14 ppi and all animals on day 28 ppi. Addition of the LT vectors to pWRGFaeGopt further enhanced this response so that 5 on 6 pigs were positive on day 14 ppi and the whole group on day 21 ppi. Consequently, the F4-specific serum IgG titer was significantly higher in the pWRGFaeGopt + LT and the pWRGFaeGopt groups on days 28, 35 and 43 and days 21, 28 and 35 ppi, respectively, than in the other groups.

F4-specific proliferation of PBMC collected at day 35 ppi was only observed in the LT supplemented group ($p < 0.05$) (Figure 2). Medium proliferation was 482 ± 235 [mean counts per minute (cpm) \pm standard error of the mean (SEM)] and was not significantly different between the groups.

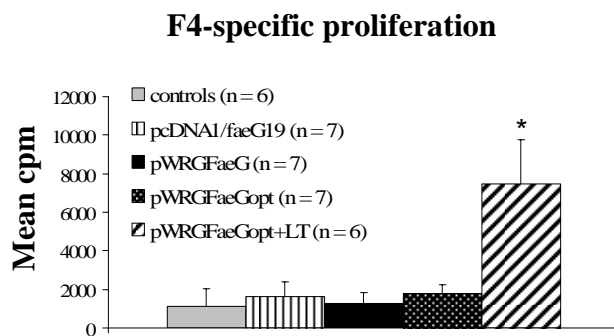


Figure 2: F4-specific proliferation of PBMC at day 35 ppi. Results are presented as cpm + SEM. * indicates significant differences ($P < 0.05$) between the pWRGFaeGopt + LT group and the other groups.

Oral booster immunization with F4 fimbriae

To demonstrate priming of the intestinal mucosal immune system by the intradermal DNA vaccination, all pigs received an oral boost with F4. This boost slightly increased the mean F4-specific IgM titer within each group, but no significant differences were observed between the groups. The F4-specific serum IgA titer increased in only 1 pig of the pWRGFaeGopt group (titer 80), 1 of the LT group (titer 15) and 3 of the pWRGFaeG group (titers ranging from 15 to 40). No significant difference was seen between groups. However, a significant increase of the F4-specific serum IgG titer did occur in the pWRGFaeGopt + LT group at day 49 ppi. At that day, a 4.5-fold increase in the mean F4-specific IgG titer was observed in this group, compared to a 2-, 1.4-, 1.1- and 1.1-fold increase in the pWRGFaeGopt, the pWRGFaeG, the pcDNA1/*faeG19* and the control group, respectively. In the latter group,

only the F4R⁺ control pigs showed this increase. Such a difference in response between F4R⁺ and F4R⁻ pigs was not observed for the FaeG DNA primed pigs.

To localize and quantify the antibody response following the F4 boost 3 pigs of each FaeG DNA vaccinated group and 2 control pigs were euthanized at day 49 or 50 ppi. One pig in each of these groups was F4R⁻ as shown in the *in vitro* villus adhesion assay. The most important difference between groups were observed in the spleen and to a lesser extent in the Mes LN (Figure 3). In the spleen, no IgG ASC could be observed for the control and the pcDNA1/*faeG19* group, while all tested pigs of the 3 pWRG groups showed IgG ASC. Furthermore, the number of F4-specific IgG ASC increased in the order pWRGFaeG, pWRGFaeGopt, pWRGFaeGopt + LT group with medians of 6, 16 and 46 ASC/5x10⁶ MC, respectively. Such a difference was not observed for IgM and IgA ASC, except for the pcDNA1/*faeG19* group which showed a higher IgM response (Figure 3).

Also in GALT, F4-specific IgG ASC could only be detected in the FaeG DNA primed animals. In the LP, high numbers of F4-specific IgG and low numbers of F4-specific IgM ASC were observed in 1 pig of the pWRGFaeG (44 and 8 ASC/5x10⁶ MC, respectively) and 1 pig of the pWRGFaeGopt group (82 and 6 ASC/5x10⁶ MC, respectively) (Figure 3). These pigs were both F4R⁺. In the Mes LN, a low F4-specific IgG response could be detected in 2 pWRGFaeG (7 and 13 ASC/5x10⁶ MC) and 2 pWRGFaeGopt + LT primed pigs (8 and 21 ASC/5x10⁶ MC), irrespective of the F4R status. IgG ASC were nearly absent (< 5 ASC/5x10⁶ MC) in the Mes LN of the other DNA primed pigs (Figure 3) and in the IPP and JPP of all pigs (data not shown). Low numbers of F4-specific IgM (0 to 12 ASC/5x10⁶ MC) and IgA ASC (0 to 14 ASC/5x10⁶ MC) could be detected in the IPP, JPP and Mes LN and in all GALT, respectively, independent of DNA priming (data not shown).

In the intestinal contents, F4-specific IgA and IgG antibodies could be detected in the duodenum of the F4R⁺ pWRGFaeG pig that showed F4-specific IgG ASC in its LP and throughout the small intestine of the 2 F4R⁺ pigs of the pWRGFaeGopt group (titers of 2 to 16, data not shown).

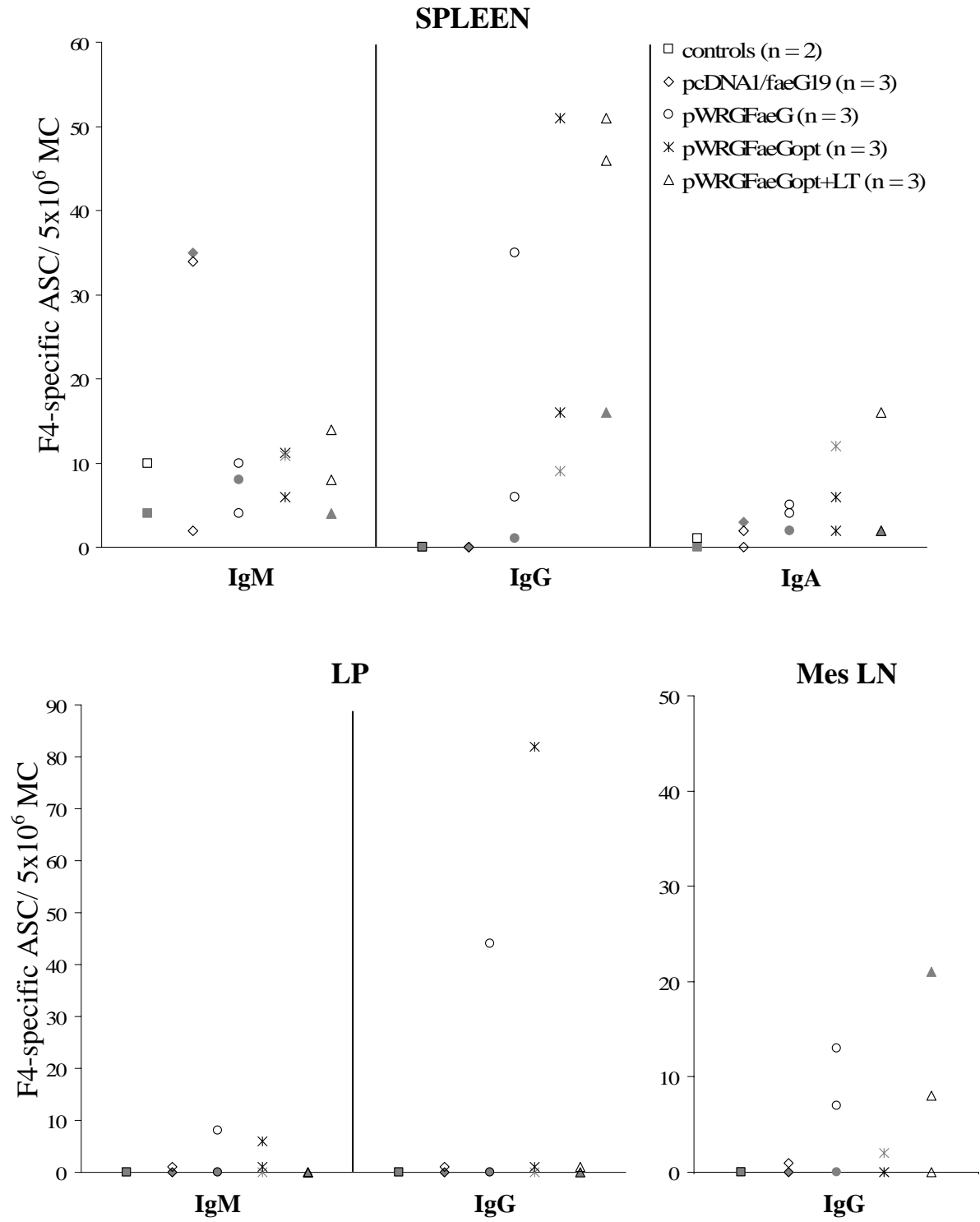


Figure 3: F4-specific ASC per 5×10^6 MC in the spleen, LP and Mes LN at day 49 or 50 ppi. Pigs were ID immunized with DNA and orally boosted with F4 fimbriae as shown in Figure 1. F4R⁻ pigs are represented in grey.

F4⁺ ETEC challenge

To determine if the induced F4-specific immune response was protective, the remaining pigs were intragastrically challenged with F4⁺ ETEC at days 51 and 52 ppi. The *in vitro* villus adhesion assay showed that 1 pig of the control group and 1 pig of the pcDNA1/*faeG*19 group were F4R⁻. Data from these pigs were not further considered.

All groups excreted F4⁺ *E. coli* after challenge. Compared to the control group, excretion was significantly reduced in the pWRGFaeGopt group at day 7 and in the pWRGFaeGopt + LT group at days 6 and 7 post challenge (pc). Moreover, the duration of the faecal F4⁺ *E. coli* excretion was reduced in the pWRGFaeGopt + LT group compared to the control group with at least 2 days ($P=0.081$) (Figure 4).

After challenge, the serum IgM titer increased 1.7-fold (day 58 ppi) in the control group, whereas almost no increase occurred in the DNA primed groups (Figure 1). Furthermore, a significant increase in mean serum IgG titer could be observed in the control group ($P=0.025$) at day 55 ppi. Insignificant increases occurred in the DNA primed groups, except for the pWRGFaeGopt and the pWRGFaeGopt + LT group. Here, the IgG titer remained significantly higher for the pWRGFaeGopt group till day 55 and for the pWRGFaeGopt + LT till day 71 ppi. The serum IgA titer slightly increased in all groups, except for the pWRGFaeGopt + LT group (Figure 1).

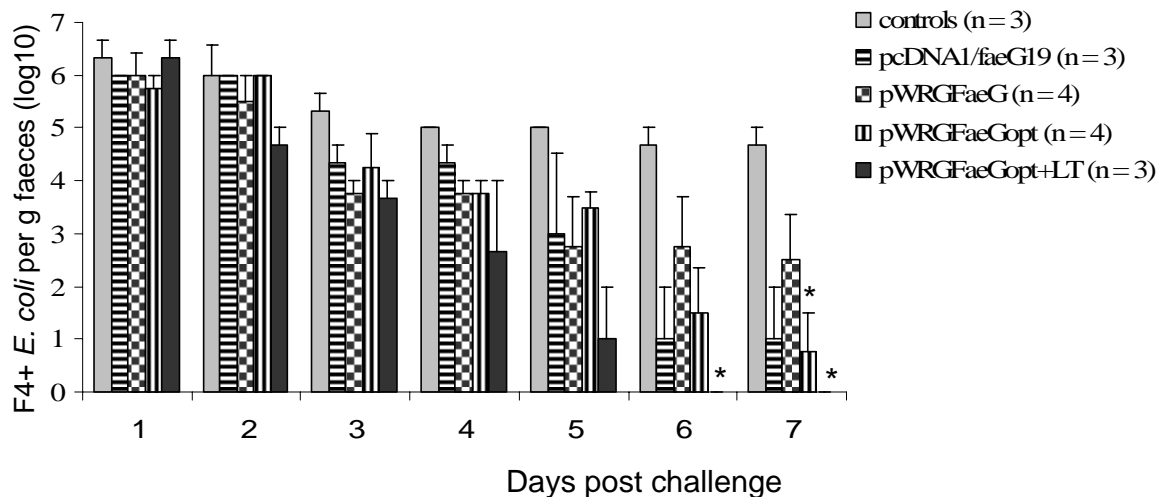


Figure 4: Mean F4⁺ *E. coli* excretion per g faeces (\log_{10} + SEM) following intragastric challenge with F4⁺ *E. coli* at days 51 and 52 ppi. Pigs were ID primed with DNA and orally boosted with F4 fimbriae as shown in Figure 1. * indicates significant differences ($P<0.05$) in excretion with the control group.

DISCUSSION

Introducing a 'Kozak' consensus sequence for translation initiation (Kozak, 1987) into prokaryotic genes has been suggested to increase their expression level in eukaryotic cells (Garmory *et al.*, 2003). Hereby, especially the presence of a purine (G/A) at the -3 and a G at the +4 position is supposed to be important (Kozak, 1997). As pcDNA1/*faeG*19 contains a T at -3 and an A at the +4 position, it was not surprising that insertion of a Kozak sequence improved the *FaeG* expression. The further improvement observed with the pWRG7079 vector in COS-7 and 293T cells may be due to the presence of the intron A, which acts as an enhancer of the CMV IE promoter (Chapman *et al.*, 1991; Wang *et al.*, 2006), and/or to the use of the BGH polyA instead of the SV40 polyA used by pcDNA1 (Montgomery *et al.*, 1993; Norman *et al.*, 1997). Presence of the tPA coding sequence could also have contributed as its AUG start codon is flanked by an A at -3 and a G at the +4 position. Furthermore, it should be mentioned that the tPA leader sequence directs *FaeG* to the secretory pathway, making it a target for *N*-glycosylation. Indeed, the *FaeG* contains 3 putative *N*-glycosylation sites. Nevertheless, although *N*-glycosylation of the *FaeG* has been demonstrated to occur in plants, it did not abolish the immunogenic character of the *FaeG* in that study (Joensuu *et al.*, 2006). Furthermore, as pWRGFaeG is far more immunogenic than pcDNA1/*faeG*19, there is no evidence that *N*-glycosylation would interfere with the immunogenicity of the *FaeG* in our study. Moreover, antibodies induced following immunization of pigs with pWRGFaeG were able to block adhesion of F4⁺ ETEC to porcine intestinal villi, indicating that they retained their biological activity (unpublished results). A positive influence of the pWRG7079 vector on expression was not observed for the PK-15 and the SK-6 cell line. Possibly, by improving transcription initiation and termination, the process of translation might have become rate limiting. Indeed, codon-optimization significantly increased the *FaeG* expression in these cell lines and in 293T cells, but not in COS-7 cells. Furthermore, COS-7 cells showed the highest increase in *FaeG* expression by using pWRG7079, suggesting that transcription rather than tRNA availability and/or mRNA stability were the rate limiting factors in these cells. Moreover, the fact that codon-optimization also appeared to be successful in the human cell line was not unexpected since the preferred codon usage in pigs is very similar to that in humans (Nakamura *et al.*, 2000).

In accordance with the increasing *FaeG* expression *in vitro*, F4-specific antibody responses were better induced in the order pcDNA1/*faeG*19, pWRGFaeG, pWRGFaeGopt. The failure of pcDNA1/*faeG*19 to induce an F4-specific serum antibody response is most likely due to

the absence of FaeG secretion, limiting the amount of antigen available for B cell priming. Furthermore, it can not be ruled out that differences in CpG motifs between the vectors as well as due to codon-optimization influenced the immunization. Indeed, it is well known that CpG added to a vaccine can have an adjuvant effect on the immunization via interaction with Toll-like receptor 9. Changes in the CpG content might thus result in changes in the adjuvant effect of the DNA vaccine. However, it should be mentioned that the potential adjuvant effect of CpG in the vaccine is still a matter of discussion in large animals. While studies in mice demonstrated enhanced humoral responses by inserting CpG motifs in the vector backbone (Ma *et al.*, 2002), the insertion of up to 88 ruminant-specific CpG motifs did not quantitatively affect the humoral response in cattle (Pontarollo *et al.*, 2002).

F4-specific lymphocyte proliferation of PBMC could only be demonstrated in the group that received the LT vectors. A positive effect of LT on cellular responses was also observed by Arrington *et al* (2002) evaluating the same vectors for their adjuvant effect in mice. In their study, augmented cellular responses were accompanied by a Th1-modulating effect and were not due to CpG motifs in the vectors, but mainly resulted from the expressed LTA and B subunits. In our study, addition of the LT vectors enhanced the serum IgG response, but abolished the serum IgA response observed following immunization with pWRGFaeGopt. These results were confirmed by an IgA capture ELISA (data not shown), eliminating the possibility that the higher IgG levels in the pWRGFaeGopt + LT group competed with binding of IgA in ELISA. Although cytokine expression profiles and IgG subclass ratios remain to be tested, a likely explanation could be the induction of a Th1-like response by addition of the LT vectors, as observed in mice. Indeed, Th1 is less favourable for IgA responses. Intradermal injection of the LT vectors was well tolerated by the pigs, as a mild local reaction could only be demonstrated in 3 out of 6 pigs. This reaction resolved by day 7 (data not shown).

The oral F4 administration induced a secondary serum IgG response in the pWRGFaeGopt and especially in the pWRGFaeGopt + LT group. This response was independent of the F4R status, which was not completely unexpected since oral F4 administration has previously been shown to prime systemic responses in both F4R⁺ and F4R⁻ pigs (Van den Broeck *et al.*, 2002). Studying the ASC in different tissues revealed that the antibody response was mainly induced systemically. Only few pigs showed an intestinal mucosal immune response with antibodies which were mainly IgG instead of IgA. Normally sIgA provides the primary defense against intestinal pathogens like ETEC. Nevertheless, priming with pWRGFaeGopt significantly reduced the faecal F4⁺ *E. coli* excretion, whereas co-administration of the LT vectors

additionally reduced the duration of faecal excretion. The reason for this partial protection is not clear but the IgG response might play a role. Indeed, a study by Yu *et al* (2002) demonstrated protection of mice against an oral LT challenge by passive infusion of serum derived anti-LT IgG. It has been demonstrated that IgG can be transported towards the intestinal lumen via the bidirectional IgG transporting neonatal Fc receptor (FcRn) (Dickinson *et al.*, 1999). Expression of the FcRn receptor has been demonstrated on the intestinal epithelium of young and of adult humans and pigs (Israel *et al.*, 1997; Stirling *et al.*, 2005). FcRn mediated IgG transport might explain the presence of F4-specific IgG in the intestinal contents of some DNA primed pigs. Once in the intestinal lumen, IgG could contribute to protection by neutralizing adhesins so inhibiting bacterial colonisation. Indeed, oral administration of milk derived anti-CFA/I IgG has protected human volunteers against a CFA/I⁺ ETEC infection (Freedman *et al.*, 1998).

In conclusion, our results showed that optimization of the expression construct and co-administration of the LT vectors strongly enhanced the immunogenicity of our FaeG DNA vaccine in pigs. Nevertheless, the ID FaeG DNA prime-oral F4 boost immunization mainly induced a systemic IgG response, failing to prevent F4⁺ *E. coli* excretion upon challenge. For the development of a vaccination strategy which can prevent postweaning diarrhea, further optimizations are required. Inclusion of other immunomodulators, like vitamin D₃, which has been shown to modulate systemic responses towards mucosal ones in pigs as well as in mice (Enioutina *et al.*, 1999, 2000, Van der Stede *et al.*, 2001, 2003, 2004) or strategies which enable delivery of the DNA vaccine to the intestinal mucosa might result in the induction of the local intestinal mucosal response that is needed.

Acknowledgements

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CHAPTER 5

Comparison of immune responses in parenteral FaeG DNA primed pigs boosted orally with F4 protein or reimmunized with the DNA vaccine

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ABSTRACT

We previously showed that an intradermal (ID) FaeG DNA prime (2x) - oral F4 protein boost immunization induces a systemic response and weakly primes a mucosal IgG response in pigs, especially when plasmid vectors encoding the A and B subunit of the *E. coli* thermostable enterotoxin (LT) are added to the DNA vaccine. In the present study, we evaluated whether addition of 1 α ,25-dihydroxyvitamin D₃ (vitD₃) to the DNA vaccine could further enhance this mucosal priming and/or modulate the antibody response towards IgA. To further clarify priming of systemic and mucosal responses by the ID DNA vaccination, we firstly compared the localization of the F4-specific antibody response in pigs that were orally boosted with F4 to that in pigs that received a third ID DNA immunization and secondly evaluated cytokine mRNA expression profiles after ID DNA vaccination. The ID DNA prime (2x) -oral F4 boost immunization as well as the 3 ID DNA vaccinations induced mainly a systemic response, with a higher response observed following the heterologous protocol. Co-administration of vitD₃, and especially of the LT vectors, enhanced this response. Furthermore, only the heterologous immunization resulted in a weak mucosal priming, which appeared to require the presence of the LT vectors or vitD₃ as adjuvants. In addition, the LT vectors strongly enhanced the FaeG-specific lymphocyte proliferation and this was accompanied by the absence of an IL-10 response. However, despite two DNA immunizations in the presence of these adjuvants and an oral F4 boost, we failed to demonstrate the secretory IgA response needed to be protective against enterotoxigenic *E. coli*.

INTRODUCTION

Enterotoxigenic *E. coli* (ETEC) that express F4 (K88) fimbriae are an important cause of diarrhoea in recently weaned piglets. The F4 fimbriae are long proteinaceous appendages mainly composed of several hundreds identical FaeG subunits. They enable the bacteria to adhere to F4-specific receptors (F4R) on the intestinal epithelium and subsequently to colonize the small intestine (Nagy *et al.*, 1985). Presence of the F4R is genetically determined and F4R negative (F4R⁻) pigs are resistant to an F4 positive (F4⁺) ETEC infection (Rutter *et al.*, 1975). It was previously shown that oral immunization of F4R⁺ weaned pigs with F4 fimbriae resulted in an antigen-specific secretory IgA (sIgA) response at the intestinal mucosa, protecting these pigs against a subsequent F4⁺ ETEC challenge (Van den Broeck *et*

al., 1999a). However, to prevent post-weaning diarrhoea, an F4-specific intestinal mucosal immune response should already be primed during the suckling period, often in the presence of F4-specific maternal antibodies. These lactogenic antibodies will hamper the use of F4 as a vaccine. Several studies have shown that DNA vaccines, in contrast to conventional vaccines, can successfully prime immune responses in the presence of maternal antibodies (Hassett *et al.*, 1997; Fischer *et al.*, 2003; Van Loock *et al.*, 2004). Therefore, we hypothesized that priming with an FaeG DNA vaccine during the suckling period combined with an oral F4 protein boost immediately after weaning could be an interesting approach. However, in a previous study a parenteral DNA prime - oral F4 boost induced a good systemic response, but was weak in priming mucosal immunity, failing to completely prevent F4⁺ *E. coli* colonization. Furthermore, addition of plasmid vectors encoding the LTA and LTB subunits to the FaeG DNA vaccine enhanced the IgG response, but did not result in the sIgA response that is desired to completely prevent an F4⁺ ETEC infection. Nevertheless, a significant reduction was obtained in the amount of F4⁺ ETEC excreted as well as in the duration of faecal F4⁺ ETEC excretion (Melkebeek *et al.*, 2007). These data suggest that the systemic DNA immunization is weak in priming mucosal responses. Previous studies showed that a systemic immunization could induce an intestinal mucosal IgA response in mice (Enioutina *et al.*, 1999, 2000) and in pigs (Van der Stede *et al.*, 2001, 2004) if 1 α ,25-dihydroxyvitamin D₃ (vitD₃) was used as adjuvant.

Therefore, we examined in the present study whether addition of vitD₃ could enhance the mucosal priming by the FaeG DNA vaccination and/or whether it could modulate the antibody response towards IgA in the ID DNA prime – oral F4 boost immunization. Furthermore, to gain insight in the way systemic and mucosal responses are primed by the ID DNA vaccination, we compared the localization of the F4-specific antibody response in pigs that were orally boosted with F4 to that in pigs that were reimmunized ID with DNA and we evaluated cytokine mRNA expression profiles in the local draining lymph nodes after the ID DNA priming.

MATERIAL AND METHODS

Animals

Forty-nine conventionally bred pigs (Belgian Landrace x Piétrain), seronegative for antibodies against F4 as determined by ELISA, were weaned at the age of 4 weeks. Subsequently, they were housed in isolation units. From one day before weaning, all animals

were orally treated with colistine (150,000 U/kg body weight; Colivet, Prodivet Pharmaceuticals, Eynatten, Belgium) during 5 successive days to prevent *E. coli* infections during the weaning period

Isolation of F4 fimbriae

F4 fimbriae were isolated from the ETEC strains GIS26, serotype 0149:K91:F4ac, LT⁺STa⁺STb⁺, and IMM01, serotype 0147:F4ac, LT⁺STb⁺ as described by Van den Broeck *et al* (1999a). The protein concentrations of the fimbrial solutions were determined by the bicinchoninic acid reaction with bovine serum albumin as a standard (ICN Biomedicals, Belgium) and the purity was assessed using a Coomassie stained 15% SDS-PAGE and the Image Master 1D prime software (Amersham Pharmacia Biotech, Belgium). GIS26 F4 fimbriae were used for the oral immunization of pigs. The IMM01 strain carries F4 fimbriae with an FaeG sequence identical to the GIS26 strain, but fimbriae isolated from this strain have a higher purity, containing no flagellin (Verdonck *et al.*, 2004). Therefore, IMM01 fimbriae were used for the FaeG-specific ELISA and ELIsot assays and, after sterilization by filtration through a 0.2 µm filter, in an FaeG-specific lymphocyte proliferation assay.

Plasmids and 1α,25(OH)₂D₃

The pcDNA3-rpGM-CSF plasmid consists of the cDNA encoding the porcine GM-CSF cloned in the pcDNA3.1zeo expression vector.

The pWRGFaeGopt vaccine was constructed as previously described (Melkebeek *et al.*, 2007). This construct consists of a codon optimized *faeG* cloned into the pWRG7079 vector behind a tPA signal sequence, allowing the extracellular secretion of the encoded FaeG. The pJV2004 and pJV2005 plasmids consist of the pWRG7054 vector encoding the A and B subunit of the thermolabile enterotoxin of *E. coli* (LT), respectively, behind a tPA signal sequence to allow their extracellular secretion (Arrington *et al.*, 2002). All plasmids were propagated in *E. coli* DH5α and large-scale purification of the plasmids was conducted by Qiagen Endofree plasmid kits (Qiagen GmbH, Germany). After determining purity and concentration by measuring the OD at 260 and 280 nm, the plasmids were stored at -20°C.

1α,25(OH)₂D₃ (vitD₃) (Sigma), was dissolved in absolute ethanol at a concentration of 200 µg/ml and stored at 4° C.

Experimental procedure

Experiment 1

A first experiment was performed to confirm the induction of an F4-specific systemic immune response by ID immunization with pWRGFaeGopt or pWRGFaeGopt in combination with the LT vectors as well as the priming of an F4-specific intestinal mucosal IgG response by ID immunization with these vectors followed by an oral F4 boost as seen in a previous study (Melkebeek *et al.*, 2007). Furthermore, we wanted to evaluate if the immunomodulating adjuvant vitD₃ could enhance the mucosal priming by the FaeG DNA vaccine and/or modulate the induced response towards an IgA response.

Hereto, 23 pigs were used (Table 1). One week postweaning, they were divided into 4 groups and were all injected intradermally (ID) at the left site of the neck with 250 µg of pcDNA3-rpGM-CSF. Seven and 28 days later (days 0 and 21 post primary immunization (ppi)), the pigs were ID immunized at this site (at the pcDNA3-rpGM-CSF injection site) with 500 µg of pWRGFaeGopt (n=7, pWRGFaeGopt group), pWRGFaeGopt supplemented with 100µg pJV2004 and 100µg pJV2005 (collectively referred to as the LT vectors) (n=7, pWRGFaeGopt + LT group), pWRGFaeGopt supplemented with 5 µg of vitD₃ (n=5, pWRGFaeGopt + vitD₃ group) or the empty pWRG7079 vector (n=4, pWRG7079 group). At day 42 ppi, 4 pigs of both the pWRGFaeGopt and the pWRGFaeGopt + LT group and 2 pigs of both the pWRGFaeGopt + vitD₃ and the pWRG7079 group received an identical ID booster immunization, whereas the remaining pigs (3 pigs of each immunized group and 2 pigs of the pWRG7079 group) were orally boosted with 1 mg F4 fimbriae diluted in 10 ml PBS during 3 successive days (day 42, 43 and 44 ppi, respectively). The animals were deprived of food and water from 3h before till 2h after the oral immunization. The F4-specific humoral immune response was analyzed weekly by determining the F4-specific IgG, IgA and IgM titer in the serum of all pigs starting from day -7 till day 48 ppi. In order to localize and quantify the antibody response, the pigs were euthanized 6 or 7 days after the third immunization (day 48 or 49 ppi, respectively) for enumeration of the F4-specific IgG, IgA and IgM antibody-secreting cells (ASC) in the lymph node draining the DNA vaccination site (cervical superficial dorsal lymph node (Cerv LN)), the mesenteric LN (Mes LN), the ileal and jejunal Peyer's patches (IPP and JPP), the jejunal lamina propria (LP) and the spleen. To evaluate the induction of an F4-specific cellular response, an F4-specific lymphocyte proliferation assay was performed on spleen mononuclear cells (MC). Since the F4 receptor (F4R) is essential for inducing an intestinal IgA response, the presence of the F4R was determined on small intestinal villi as earlier described.

Table 1: Experimental design of experiment 1

Group	Day -7	DNA vaccine (days 0 and 21 ppi)	Third immunization (day 42 ppi)	Total number of pigs	F4R ⁺ pigs	F4R ⁻ pigs
pWRG7079 (control)	pcDNA3-rpGM-CSF	pWRG7079	pWRG7079 oral F4	2 2	1 1	1 1
pWRGFaeGopt (FaeG)	pcDNA3-rpGM-CSF	pWRGFaeGopt	pWRGFaeGopt Oral F4	4 3	3 1	1 2
pWRGFaeGopt+LT (LT)	pcDNA3-rpGM-CSF	pWRGFaeGopt+LT	pWRGFaeGopt+LT oral F4	4 3	3 1	1 2
pWRGFaeGopt+vitD ₃ (vitD ₃)	pcDNA3-rpGM-CSF	pWRGFaeGopt+vitD ₃	pWRGFaeGopt+ vitD ₃ oral F4	2 3	1 2	1 1

Experiment 2

A second experiment was performed to examine the induction of cellular immune responses (FaeG-specific lymphocyte proliferation and cytokine mRNA expression) in the local draining lymph node (Cerv LN) following priming with the FaeG DNA vaccine and to evaluate the influence of the intradermal LT vectors or vitD₃ on these cellular responses. Hereto, 26 pigs were used (Table 2). One week after weaning, 6 pigs were ID injected in the neck with sterile PBS (PBS group) and the other pigs with 250µg of pcDNA3-rpGM-CSF. Seven and 28 days later (days 0 and 21 ppi), the pigs were ID injected at the same site with PBS (pigs from the PBS group) or 500 µg of pWRGFaeGopt (n=5, pWRGFaeGopt group), pWRGFaeGopt supplemented with 100µg of each LT vector (n=6, pWRGFaeGopt + LT group), pWRGFaeGopt supplemented with 5 µg of vitD₃ (n=5, pWRGFaeGopt + vitD₃ group) or pWRG7079 (n=4, pWRG7079 group). The pigs were euthanized 24 or 48 hours after the second immunization (psi) as shown in Table 2 and for each pig the cervical superficial dorsal LN was collected at the left site (Cerv LN) and the right site (control Cerv LN) of the neck to analyze cytokine expression profiles. Cerv LN MC isolated at 48 hours psi were also used in an F4-specific lymphocyte proliferation assay.

Table 2: Experimental design of experiment 2

Group	Total number of pigs	Day -7	Days 0 and 21 ppi	Number of pigs at 24h psi	Number of pigs at 48h psi
PBS	6	PBS	PBS	3	3
pWRG7079	4	pcDNA3- rpGM-CSF	pWRG7079	2	2
pWRGFaeGopt	5	pcDNA3- rpGM-CSF	pWRGFaeGopt	3	2
pWRGFaeGopt+LT	6	pcDNA3- rpGM-CSF	pWRGFaeGopt +LT	2	4
pWRGFaeGopt+vitD3	5	pcDNA3- rpGM-CSF	pWRGFaeGopt +vitD3	2	3

In both experiments, plasmids were diluted in 1 ml sterile PBS and both the ID injection with pcDNA3-rpGM-CSF as the ID immunizations were performed by multiple injections. Euthanasia of all pigs was performed by intravenous injection of pentobarbital (24 mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) followed by exsanguinations.

Spleen, Cerv LN, Mes LN, JPP, IPP and LP MC

MC were isolated from the spleen, Cerv LN, Mes LN (jejunal and ileal), JPP, IPP and LP as described by Verdonck *et al.* (2002) and finally resuspended in leukocyte medium [RPMI-1640 (GIBCO BRL) supplemented with penicillin (100 IU/ml) (GIBCO BRL), streptomycin (100 µg/ml) (GIBCO BRL), kanamycin (100 µg/ml) (GIBCO BRL), L-glutamin (200 mM) (GIBCO BRL), sodiumpyruvate (100mM) (GIBCO BRL), non-essential aminoacids (100 mM) (GIBCO BRL), 2-mercaptoethanol (5×10^{-5} M) (GIBCO BRL) and either 5% serum of a colostrum-deprived pig for the lymphocyte proliferation assay or 10% FCS for the ELISPOT assay]. Cerv LN MC were also resuspended in TRIzol Reagent (Invitrogen, Merelbeke, Belgium) (Cerv LN) to analyze cytokine mRNA expression.

RNA extraction and RT-PCR

RNA was extracted from 10^7 MC/sample using TRIzol Reagent following the manufacturer's protocol. Reverse transcription was performed as described by Verfaillie *et al.* (2005). The oligonucleotide primers described by Verfaillie *et al.* (2005) were used for the detection of the porcine cytokines IL-2, IL-4, IL-6, IL-10, TGF- β , IFN- γ , TNF- α and of cyclophilin cDNA. Oligonucleotide primers used for the detection of β -actin and GAPDH cDNA were designed from the published nucleic acid sequences available from the GenBank/EMBL databases using the primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 3).

Real-time PCR reactions were carried with the LightCycler and the LightCycler-faststart DNA Master SYBR Green I kit (Roche, Mannheim, Germany). The reactions for IL-2, IL-4, IL-6, IL-10, TGF- β , IFN- γ , TNF- α and of cyclophilin were performed as described by Verfaillie *et al.* (2005). For β -actin and GAPDH, the reaction mixture was made following the manufacturer's protocol using 3 mM MgCl₂. After heating the samples at 95° C for 10 min, the temperature cycling consisted of denaturation at 94° for 15 s, annealing at 53° C (GAPDH) or 60° C (β -actin) for 5 s, and extension at 72° C for 14 s. For β -actin 35 cycles and for GAPDH 40 cycles were performed. Fluorescence acquisition was measured at 85°C in single mode. Melting curve analysis was done at 65-98°C with continuous fluorescence acquisition. As an additional control of specificity, PCR products were subjected to agarose gel electrophoresis. Quantification occurred using external standards of cDNA and the copy number of each sample transcript was determined with the aid of the LightCycler software. Cyclophilin, GAPDH and β -actin were used as constitutively expressed reference genes and a normalization factor (NF) was calculated as the geometric mean of the copy numbers of these

Table 3 :Oligonucleotide primers and fragment length of PCR products for different porcine cytokines

cytokine	Primers (5'-3')	Length PCR fragment	Genbank accession number
IL-1 α	(S) TGCCAGCTATGAGCCACTTCC (AS) TGACGGGTCTCGAATGATGCT	336	X52731
IL-2	(S) GATTACAGTTGCTTTTGAAG (AS) GTTGAGTAGATGCTTTGACA	338	X56750
IL-4	(S) TACCAGCAACTTCGTCCA (AS) ATCGTCTTTAGCCTTTCCAA	311	F68330
IL-6	(S) ATGAGAATCACCACCGGTCTTG (AS) TGCCCCAGCTACATTATCCGA	310	M86722
IL-10	(S) CCATGCCCAGCTCAGCACTG (AS) CCCATCACTCTCTGCCTTCGG	295	L20001
IFN- γ	(S) ATGTACCTAATGGTGGACCTC (AS) CTCTCTGGCCTTGGAACATAG	360	X53085
TGF- β	(S) GACCCGCAGAGAGGCTATAG (AS) GAGCCAGGACCTTGCTGTAC	399	Y00111
TNF- α	(S) CAAGGACTCAGATCATCGTCTCA (AS) CATACCCACTCTGCCATTGGA	100	X54859
Cyclophilin	(S) TAACCCACCGTCTTCTT (AS) TGCCATCCAACCACTCAG	368	F14571
B-actin	(S) ACGTGGACATCAGGAAGGAC (AS) ACATCTGCTGGAAGGTGGAC	210	U07786
GAPDH	(S) GGGCATGAACCATGAGAAGT (AS) AAGCAGGGATGATGTTCTGG	230	AF017079

(S) : sense ; (AS) : antisense

3 genes to determine the uniformity of the reverse transcription and as a reference for quantification of cytokine mRNA. The relative amounts of cytokine-expression were calculated as a ratio of the copy number of the target cytokine over the NF and results are presented as the ratio of the relative amount of the target cytokine in the immunized Cerv LN over the relative amount of the target cytokine in the control Cerv LN $(= (\text{copy number of target cytokine in the Cerv LN} / \text{NF in the Cerv LN}) / (\text{copy number of target cytokine in the control Cerv LN} / \text{NF in the control Cerv LN}))$.

FaeG-specific lymphocyte proliferation assay.

To determine the FaeG-specific lymphocyte proliferation, a proliferation assay as described by Van der Stede *et al* (2003) was performed on spleen or Cerv LN MC by adding F4 fimbriae (final concentration of 15 µg/ml), medium (negative control) or concanavalin A (ConA, final concentration of 10 µg/ml, positive control) to the wells of a 96-well plate (Cellstar, Greiner bio-one, Wommel, Belgium) containing 5×10^5 cells/well (final volume of 200 µl). After 72 h at 37 °C in 5% CO₂, cells were pulse-labelled with ³H-thymidine (1 µCi/well) (Amersham ICN, Bucks, UK) and 18 h later, they were harvested onto glass fibre filters. The radioactivity incorporated into the DNA was measured with a β-scintillation counter (Perkin-Elmer, Life Science, Brussels, Belgium).

ELISA for FaeG-specific serum IgG, IgA and IgM antibodies

FaeG-specific titers were determined by an indirect ELISA on serum sampled as described by Van den Broeck *et al.* (1999b). Hereto, the wells of a 96-well microtitre plate (NUNC, Polysorb Immuno Plates, Roskilde, Denmark) were coated with IMM01 F4 fimbriae at a concentration of 5 µg/ml in PBS. After 2 h incubation at 37° C, the remaining binding sites were blocked overnight at 4° C with PBS supplemented with 0.2% Tween® 80 (Merck Eurolab, Leuven, Belgium). Subsequently, the sera were added for 1 h at 37° C in series of 2-fold dilutions in ELISA dilution buffer (PBS, pH 7.4 + 0.2 % Tween® 20 + 3 % BSA), starting at a dilution of 1/10. Then, optimal dilutions of anti-swine IgM-, IgA- or IgG-specific MAb (28.4.1, 27.8.1 and 23.3.1b, respectively, Van Zaane and Hulst, 1987) in ELISA dilution buffer were added to the wells for 1 h at 37° C. Thereafter, rabbit anti-mouse HRP-conjugated serum (Dako, Denmark) optimally diluted in ELISA dilution buffer and supplemented with 2% pig serum was added for 1 h at 37° C. Finally, ABTS (Roche Diagnostics, Brussels, Belgium), containing H₂O₂, was added and after 30 minutes incubation at 37° C, the optical density was measured spectrophotometrically at 405 nm (OD₄₀₅). Between each incubation

step, the plates were washed 3 times with ELISA washing buffer (PBS, pH 7.4 + 2% Tween 20). Cut-off values were calculated as the mean OD₄₀₅ of all sera (dilution 1/10) on day 0 ppi increased with three times the standard deviation and were 0.16, 0.23 and 0.63 for IgA, IgG and IgM, respectively. The antibody titer was the inverse of the highest dilution which still had an OD₄₀₅ higher than the calculated cut-off values.

Elispot assay for FaeG-specific IgG, IgA and IgM ASC

F4-coated plates were prepared as described for the ELISA. Thereafter, MC suspensions at a concentration of 10^7 cells/ml were added (100 μ l/well) and plates were incubated for 14 h at 37° C in a humidified 5% CO₂ atmosphere. Subsequently, the cells were removed by six washes with ELISA washing buffer whereafter wells were sequentially incubated for 1 h at 37° C with the anti-swine IgM-, IgA- or IgG-specific MAb and with a rabbit anti-mouse HRP-conjugated serum. Between these steps, the plates were washed 3 times with ELISA washing buffer. Subsequently, a substrate solution, consisting of 4 volumes of 3-amino-9-ethylcarbazole (AEC) (Sigma) working solution (0.67 ml AEC stock solution (0.4% in dimethylformamide) in 10 ml Na acetate (0.1 M, pH 5.2) + 10 μ l 30% H₂O₂) and 1 volume of 3% low-melting-point agarose (BIOzym, Landgraaf, The Netherlands) was added. After overnight incubation in the dark at room temperature, spots were counted with an inverted microscope. For each MC suspension, spots in 5 wells (10^6 MC/well) were counted to obtain the number of isotype-specific ASC/ 5×10^6 MC. Results are presented as the mean number of ASC per 5×10^6 MC \pm SEM.

Statistical analysis

Statistical analysis was done using SPSS 12.0 for Windows. Analysis of differences between the groups in log₂ serum antibody titers was done using General Linear Model (Repeated Measures Analysis of Variance) using the Bonferoni adjustment for multiple comparisons. Differences between groups in lymphocyte proliferation and in relative increase in cytokine expression at the immunization site were tested for statistical significance with the Kruskal-Wallis Test. $P \leq 0.05$ was considered as statistically significant.

RESULTS

A. EXPERIMENT 1: FaeG-specific antibody responses following DNA vaccination

F4R characterization of the pigs

As shown in Table 1, the *in vitro* villous adhesion assay demonstrated that 6 of the 11 pigs that were orally boosted with F4 and 4 of the 12 pigs that were ID boosted with DNA lacked the F4R.

FaeG-specific serum antibody response following 2 intradermal DNA vaccinations

Pigs were immunized ID with DNA at days 0 and 21 ppi. Since pre-injection with pcDNA3-rpGM-CSF enhances both humoral and cellular responses in pigs (Melkebeek *et al.*, 2006), all pigs received an ID injection with this plasmid seven days before the first immunization (Table 1).

FaeG-specific serum IgG was already observed after 1 immunization with pWRGFaeGopt and addition of LT enhanced this IgG response (Figure 1). VitD₃ was a less powerful adjuvant, enhancing the IgG response to a lesser extend. Control animals vaccinated with pWRG7079, did not show an IgG response. Consequently, the mean FaeG-specific IgG titer was significantly higher in the pWRGFaeGopt + LT than in the pWRGFaeGopt and the pWRG7079 group and in the pWRGFaeGopt + vitD₃ group than in the pWRG7079 group from 7 days after the second immunization (day 28 ppi) till the third immunization (day 42 ppi).

Levels of FaeG-specific IgA and IgM antibodies remained very low (mean titers ≤ 18 in all groups).

FaeG-specific responses following an oral F4 boost or a third ID DNA vaccination

To determine if the ID DNA vaccination induced an FaeG-specific intestinal mucosal antibody response or primed the gut-associated lymphoid tissues (GALT), pigs were either immunized a third time with DNA (day 42 ppi) or obtained an oral boost with 1 mg F4 fimbriae during 3 successive days (day 42, 43 and 44 ppi) (Table 1). Mucosal and systemic responses were analyzed 6 or 7 days later.

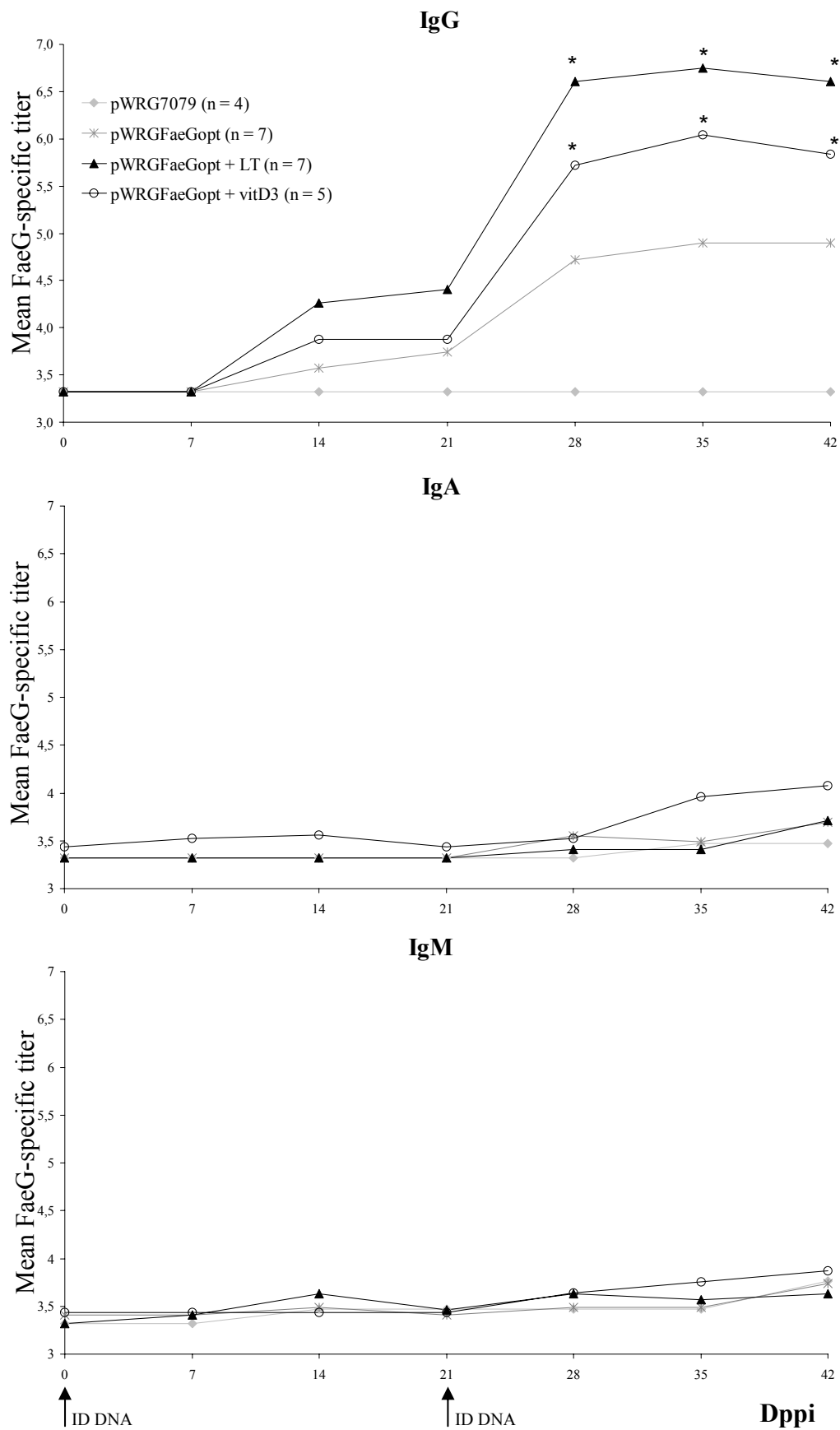


Figure 1: FaeG-specific serum IgG, IgA and IgM titers following 2 ID immunizations with pWRGFaeGopt (n=7), pWRGFaeGopt + LT (n=7), pWRGFaeGopt + vitD3 (n=5) or pWRG7079 (controls, n=4) (days 0 and 21 ppi, black arrows). Significant differences ($P<0.05$) between the pWRGFaeGopt + LT group and the pWRGFaeGopt and the control group and between the pWRGFaeGopt + vitD₃ group and the control group are indicated with *. Results are presented as means \pm SEM.

The FaeG-specific serum antibody response

Both the oral F4 boost and the ID DNA vaccination further enhanced the FaeG-specific serum IgG titers in all the FaeG DNA primed groups, irrespective of the F4R status of the animals, whereas the pWRG7079 animals remained negative (Figure 2).

None of the groups showed increased FaeG-specific serum IgA or IgM titers following the DNA or the F4 boost (data not shown).

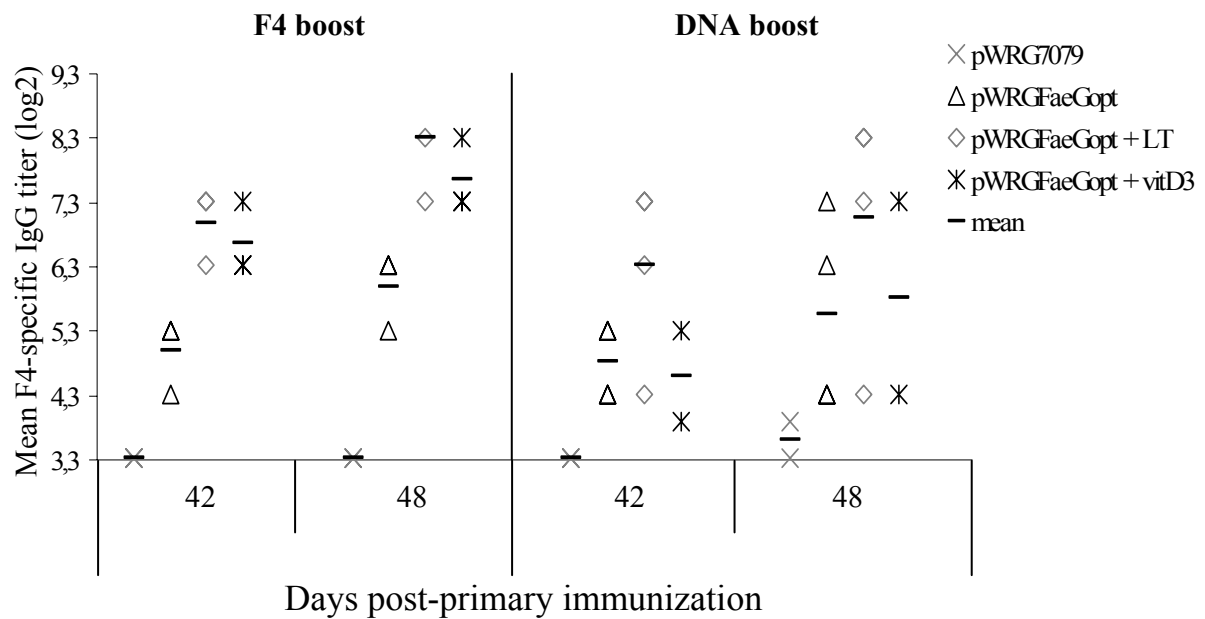


Figure 2: Mean FaeG-specific serum IgG titer at the day of the third immunization (day 42 ppi) and 6 days later (day 48 ppi). Pigs were immunized as shown in Table 1.

Localization of the FaeG-specific antibody response

FaeG- specific ASC were mainly detected in the Cerv LN (Figure 3) and to a lesser extend in the spleen (Figure 4). In the control group, no FaeG-specific ASC were identified in the Cerv LN whereas a background of FaeG-specific IgM was identified in the spleen after the third injection with pWRG7079 (negative controls, 16-21 ASC/ 5×10^6 MC) as well as after the oral F4 immunization (18-44 ASC/ 5×10^6 MC).

In all FaeG DNA vaccinated groups, the third ID DNA vaccination induced mainly FaeG-specific IgG (7-305 ASC/ 5×10^6 MC) and to a lesser extend IgM ASC (3-92 ASC/ 5×10^6 MC) in the Cerv LN whereas mainly IgM (15-209 ASC/ 5×10^6 MC) and to a lesser extend IgG ASC (0-39 ASC/ 5×10^6 MC) were induced in the spleen. Moreover, the numbers of FaeG-specific ASC in the Cerv LN and the spleen were comparable for the three FaeG DNA vaccinated groups. For FaeG-specific IgA, lower numbers of ASC could only be detected in the Cerv LN of most animals in all vaccinated groups (0-29 ASC/ 5×10^6 MC).

In the pWRGFaeGopt group, the oral F4 boost induced comparable FaeG-specific antibody responses in the Cerv LN and the spleen as the third ID DNA immunization. Indeed, similar mean numbers of FaeG-specific IgG ASC were detected in the Cerv LN of orally and ID boosted pigs (122 and 131 ASC/ 5×10^6 MC, respectively). In addition, also mainly FaeG-specific IgM ASC were found in the spleen of the orally boosted pigs of this group. In the pWRGFaeGopt + LT and the pWRGFaeGopt + vitD₃ group however, the oral F4 seemed to “boost” the systemic antibody response stronger than the third ID DNA immunization did. Indeed, in the Cerv LN of these groups, larger numbers of FaeG-specific IgG ASC were detected in the orally boosted pigs (143-696 and 69-854 ASC/ 5×10^6 MC for the pWRGFaeGopt + LT and the pWRGFaeGopt + vitD₃ group, respectively) than in pigs that were immunized 3 times with DNA. Moreover, the FaeG-specific ASC in the spleen of the orally boosted pigs were mainly IgG (39-96 and 28-53 ASC/ 5×10^6 MC for the pWRGFaeGopt + LT and the pWRGFaeGopt + vitD₃ group, respectively), whereas it was mainly IgM following the third DNA immunization. After the oral F4 boost, FaeG-specific IgA ASC could not be detected in the Cerv LN. However, few FaeG-specific IgA ASC could be detected in the spleen of all three FaeG DNA primed groups (0 to 4 ASC/ 5×10^6 MC, respectively).

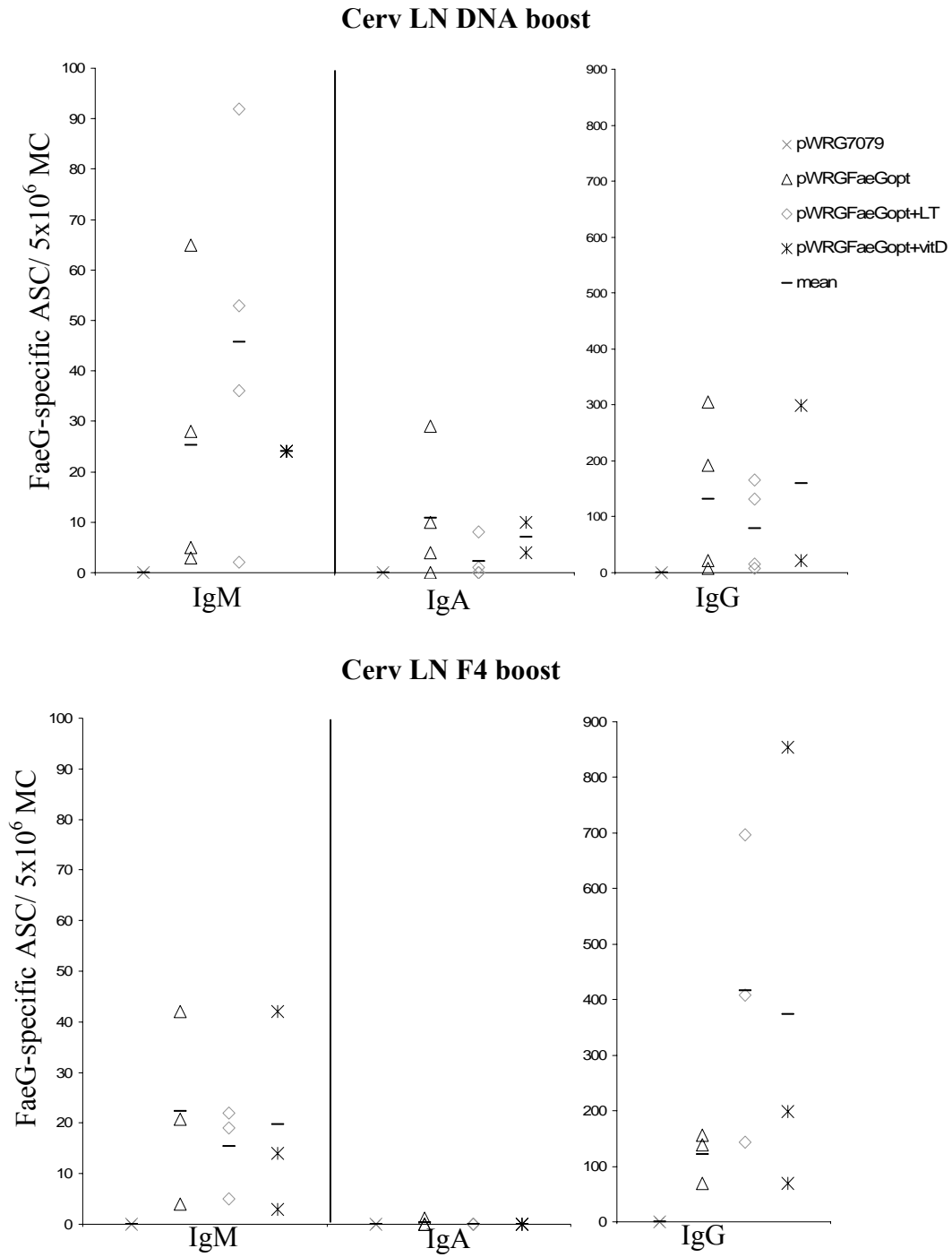


Figure 3: FaeG-specific IgM, IgA and IgG ASC/5x10⁶ MC in the Cerv LN 6 or 7 days (day 48 or 49 ppi) after the third ID DNA vaccination or the oral F4 boost. Pigs were immunized as shown in Table 1.

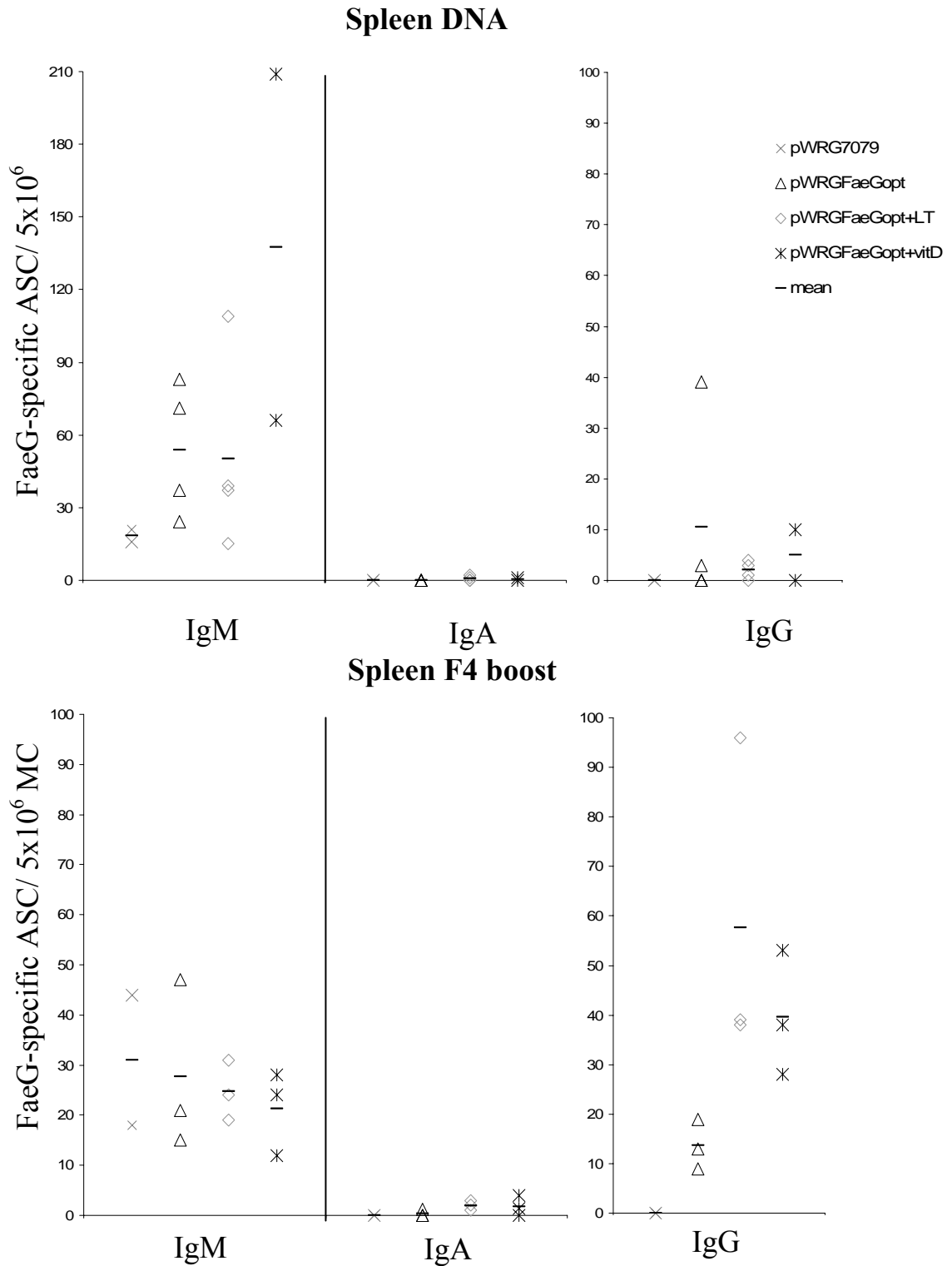


Figure 4: FaeG-specific IgM, IgG and IgA ASC/ 5×10^6 MC in the spleen 6 or 7 days (day 48 or 49 ppi) after the third ID DNA vaccination or the oral F4 boost. Pigs were immunized as shown in Table 1.

In the GALT, low numbers of IgA and IgM ASC could be detected in the orally boosted pigs of the pWRG7079 control group (up to 3 and 12 ASC/ 5×10^6 MC, respectively) whereas higher numbers were seen in some FaeG DNA primed orally boosted pigs. Indeed, high numbers of IgM ASC could be detected in the IPP, JPP and Mes LN of one F4R-positive pig primed with pWRGFaeGopt + vitD₃ (10-173 ASC/ 5×10^6 MC). An improved intestinal IgM response by DNA priming could also be observed in the LP of two F4R-negative pigs of the pWRGFaeGopt + LT group (21 and 35 ASC/ 5×10^6 MC), and in the IPP and JPP of one of these pigs and of one pig of the pWRGFaeGopt group (up to 31 and 28 ASC/ 5×10^6 MC, respectively). For these pigs, the numbers of IgA ASC were between 0 and 7 ASC/ 5×10^6 MC in the IPP, JPP and Mes LN and up to 25 ASC/ 5×10^6 MC in the LP.

The oral F4 boost seemed to weakly induce an IgG response in the GALT of DNA primed pigs. Indeed, IgG ASC could be detected in the GALT of some of the FaeG DNA primed pigs, whereas none of the control pigs showed IgG ASC in their GALT (Figure 5). However, only in the F4R-positive pig of the pWRGFaeGopt + vitD₃ group with high numbers of intestinal IgM ASC, the numbers of FaeG-specific IgG ASC were consistently high in all examined GALT (22-346 ASC/ 5×10^6 MC). In all other DNA primed pigs which received an oral F4 boost, except for one pig of the pWRGFaeGopt group, the numbers of IgG ASC were between 1 and 14 in the Mes LN. This was irrespective of the F4R status of the animals and occurred in higher numbers in both adjuvanted groups. In the other GALT, FaeG-specific IgG ASC could be detected in low numbers (1 to 4 ASC/ 5×10^6 MC) (Figure 5). This was mainly seen in the pWRGFaeGopt + LT group and more sporadically in both other DNA primed groups.

After three ID DNA immunizations, none of the pigs showed a clear FaeG-specific IgG, IgA or IgM response in their GALT (data not shown).

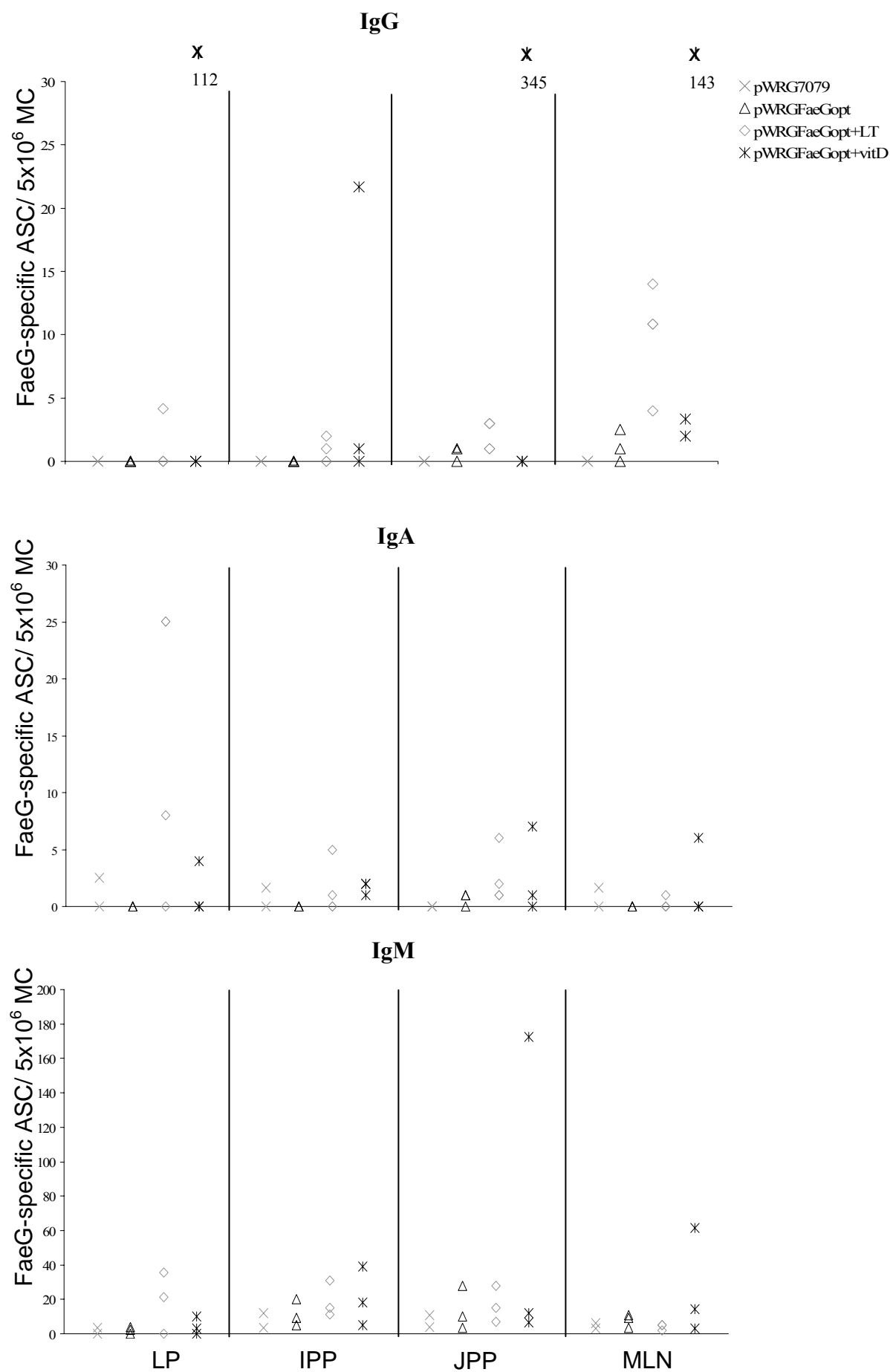


Figure 5: FaeG-specific IgG, IgA or IgM ASC/ 5×10^6 MC in the LP, IPP, JPP and MLN 6 or 7 days (day 48 or 49 ppi) after the oral F4 boost. Pigs were primed by 2 ID DNA immunizations as shown in Table 1.

B. EXPERIMENT 2: Cellular responses following DNA vaccination

Cytokine expression profiles in the local draining lymph node

To assay cytokine mRNA expression profiles following DNA vaccination, induction of IL-1 α , IL-6, TNF- α (pro-inflammatory cytokines), IL-2, IFN- γ (Th1-like cytokines), IL-4, IL-10 (Th2-like cytokines) and TGF- β (Th3-like cytokine) mRNA expression was evaluated 24 and 48 h psi (Figure 6, Table 2).

At 24h psi, a proinflammatory cytokine mRNA expression could already be observed after injection of the empty pWRG7079 vector for TNF- α , IL-1 and IL-6 (1.6-, 3.2- and 9.4-fold, respectively) and to a lesser extend after PBS injection for IL-1 and IL-6 (1.5- and 6.7-fold increase, respectively). Only the IL-6 mRNA expression was increased more strongly by the FaeG expression (pWRGFaeGopt group, 18.3-fold). A similar increase in IL-6 mRNA expression could also be observed in the pWRGFaeGopt + vitD₃ group (14.5-fold), but not in the pWRGFaeGopt + LT group, as the mean increase in IL-6 mRNA expression in this group (3.7-fold) was even lower than in the control groups. At 48h psi, the IL-6 mRNA expression was already strongly decreased, whereas the IL-1 mRNA expression was increased in the vitD₃ group. TNF- α mRNA expression remained similar in most groups.

For IL-10, IFN- γ and TGF- β mRNA, effects were most pronounced 48h psi. Expression of TGF- β mRNA expression was increased following FaeG DNA vaccination and addition of LT and vitD₃ enhanced this increase. A strong increase in IL-10 mRNA expression could be observed for one pig of the pWRG7079, the pWRGFaeGopt and the pWRGFaeGopt + vitD₃ group, whereas none of the pigs from the LT adjuvanted groups showed this increase. For IFN- γ , a strong increase in mRNA expression could only be observed for one pig of each adjuvanted group.

IL-2 and IL-4 mRNA were hardly detected and are therefore not shown.

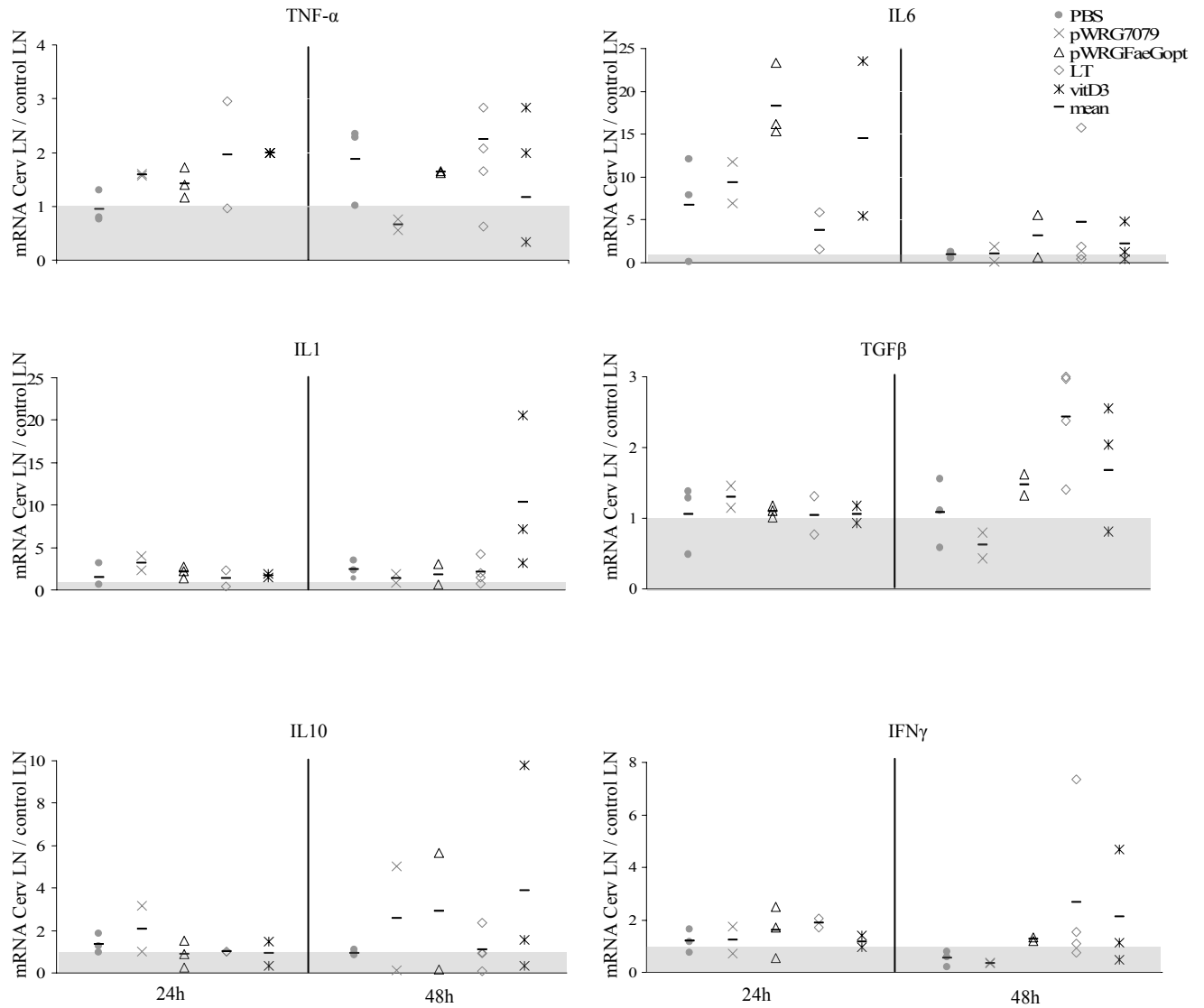


Figure 6: Cytokine mRNA expression (TNF- α , IL-6, IL-1, TGF- β , IL-10 and IFN- γ) at 24 and 48h psi. Pigs are immunized as shown in Table 2. Increases in cytokine mRNA expression by ID DNA vaccination are given as a ratio ($=$ (copy number of target cytokine in the Cerv LN / NF in the Cerv LN) / (copy number of target cytokine in the control Cerv LN / NF in the control Cerv LN))

FaeG-specific lymphocyte proliferation

To analyze cellular (memory) immune responses following ID DNA vaccination, MC isolated from the Cerv LN 48h psi or from the spleen 6 or 7 days after the third immunization (pigs from experiment 1) were restimulated *in vitro* with F4 (Figure 7).

At 48h psi, a strong FaeG-specific proliferation could only be observed in MC from the Cerv LN of 3 on 4 pWRGFaeGopt + LT (cpm between 36921 and 134394) and 1 on 3

pWRGFaeGopt + vitD₃ vaccinated pigs (73606 cpm). Interestingly, for each pig that showed a strong increase in IL-10 mRNA expression, the FaeG-specific proliferation was decreased more than 1.7 fold compared to spontaneous medium proliferation (data not shown).

Also for spleen cells isolated after three DNA immunizations, FaeG-specific lymphocyte proliferation was best induced in the pWRGFaeGopt + LT group. Indeed, a clear FaeG-specific proliferation (cpm > 5000) could be observed for 3 on 4 pigs of this group (cpm between 8420 and 23167), compared to 1 on 4 pigs of the pWRGFaeGopt and 1 on 2 pigs of the pWRGFaeGopt + vitD₃ group (cpm of 7758 and 8254, respectively). Moreover, in both the Cerv LN after 2 DNA immunizations and the spleen after 3 ID DNA vaccinations, the mean cpm value was significantly higher in the pWRGFaeGopt + LT group than in the pWRGFaeGopt group. After the oral F4 boost, a clear FaeG-specific proliferation was only seen in 1 on 3 pigs of the pWRGFaeGopt + LT group (18483 cpm).

Spontaneous proliferation was 2260 ± 431 for the Cerv LN and 1888 ± 213 for the spleen.

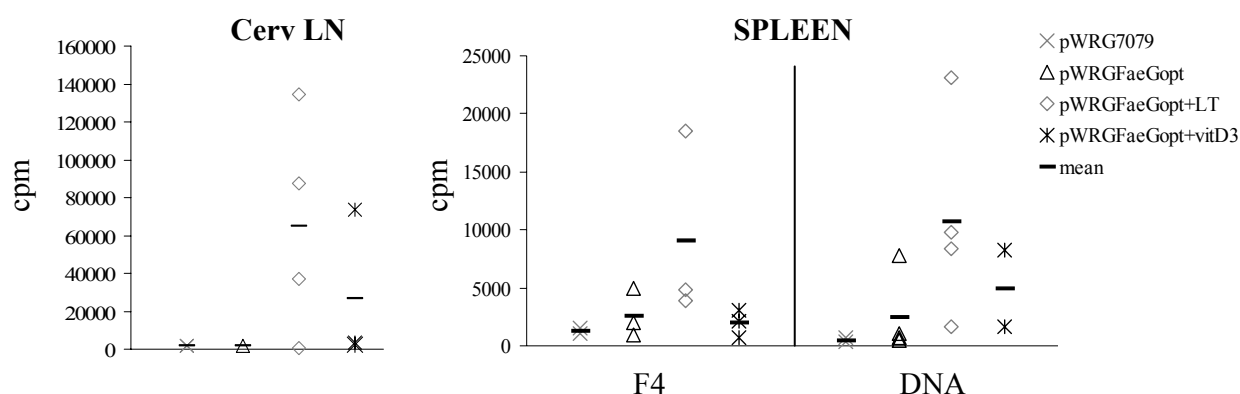


Figure 7: FaeG-specific proliferation of Cerv LN MC at 48h psi (A) and spleen MC 6 or 7 days (day 48 or 49 ppi) after the third immunization. Pigs were immunized as shown in Table 2 and Table 1, respectively. * indicates a significant difference between the pWRGFaeGopt + LT group and all other groups ($P \leq 0.05$)

DISCUSSION

In a first experiment, a heterologous ID pWRGFaeGopt DNA prime (2x)- oral F4 protein boost immunization and a homologous ID pWRGFaeGopt DNA vaccination (3x) were compared for their capacity to induce systemic and mucosal responses in pigs. Although ID DNA vaccination efficiently induced a serum IgG response, a clear FaeG-specific serum IgA

or IgM response could not be observed in the present study. This was in contrast to our previous data where a low FaeG-specific IgA response could additionally be observed after ID pWRGFaeGopt immunization, but not when the LT vectors were added (Melkebeek *et al.*, 2007). These contradicting findings might result from differences in origin of the pigs. Indeed, individual variation in immune responses, including Ig isotype responses, has been described for outbred pigs and has been attributed to differences in genetic background and environmental factors (Crawley *et al.*, 2003, Wilkie and Mallard, 1999). A second reason could be an unnoticed minor contact with F4. Indeed, it has been described that intestinal priming can result in an IgA response following parenteral immunization (Svennerholm *et al.*, 1980). Although farms were carefully selected for absence of F4⁺ ETEC related problems, litters were tested to be F4-seronegative and pigs were orally treated with colistine to prevent infection during the weaning period, a risk for such a contact can not absolutely be excluded.

Our inability to demonstrate a serum IgM response is likely due to the high cut off value in the IgM ELISA. An IgM background is often observed when young animals are tested. This is likely non-specific as even for gnotobiotic piglets, cut off values near 0.4 could be observed in F4-specific serum IgM ELISA (recent unpublished data from our lab). Furthermore, also in lymphoid tissues, a background of IgM ASC is often observed (Van den Broeck *et al.*, 1999b; Verdonck *et al.*, 2002; Snoeck *et al.*, 2006a), even for F4R⁻ pigs which are resistant to F4⁺ ETEC infection.

After both the third ID DNA immunization and the oral F4 boost, antibody responses were mainly located systemically. After the ID DNA boost, responses in the adjuvanted groups were not better than those in the pWRGFaeGopt group, suggesting that other tissues, such as the bone marrow, might account for the higher serum IgG titer in these groups. Indeed, a study by Bianchi *et al.* (1999) demonstrated that the bone marrow is the major site of IgG production in pigs, containing 40% of all IgG secreting cells.

The presence of low numbers of IgA ASC in the Cerv LN following three intradermal DNA vaccinations is in line with our previous data indicating that ID DNA vaccination preceded by GM-CSF DNA injection primes a serum IgA response that can be boosted systemically (Melkebeek *et al.*, 2006) but not mucosally (Melkebeek *et al.*, 2007). An IgA response following systemic immunization was also seen in earlier studies in pigs (Van der Stede *et al.*, 2002) and was more efficiently induced by immunizing in the neck compared to the back. The dorsal superficial cervical LN draining the neck region is connected to lymph nodes draining mucosal sites (mandibular and medial retropharyngeal LN draining tonsils and

nasal mucosa), which might be more beneficial for the induction of IgA (Vanderpooten *et al.*, 1997).

Compared to the ID DNA boost, the oral F4 boost was clearly more immunogenic for the adjuvanted groups, as judged by an enhanced isotype switching towards IgG in the spleen and increased numbers of IgG ASC in the Cerv LN. This is in agreement with other studies demonstrating that prime-boost immunizations by alternating routes and/or immunization forms are as immunogenic or more immunogenic than homologous prime-boost immunizations (Baca-Estrada *et al.*, 2000; McCluskie *et al.*, 2002, Lauterslager *et al.*, 2003). As observed previously (Melkebeek *et al.*, 2007), boosting of systemic responses in parenterally primed pigs by oral F4 administration appeared to be independent of the F4R status of the pigs.

Besides inducing a systemic response, parenteral immunization, for example with live attenuated pathogens (Coffin *et al.*, 1995) or in the presence of immunomodulating adjuvants like vitD₃, enterotoxins or chemical enhancers of cAMP (Enioutina *et al.*, 1999, 2000; Van der Stede *et al.*, 2004) has also been demonstrated to induce IgA ASC in the GALT. In our study however, an intestinal mucosal antibody response was nearly undetectable following ID DNA immunization. After the oral boost, the intestinal FaeG-specific IgA and IgM response were variable and an improvement by DNA priming could only be observed for a few pigs. In line with our previous results (Melkebeek *et al.*, 2007), IgG ASC could be detected in the Mes LN of the DNA primed and especially of the adjuvanted groups. Moreover, although the presence of the F4R is required to induce IgA and IgM ASC in the Mes LN and other GALT of naïve pigs following multiple oral F4 administrations (Van den Broeck *et al.*, 1999a), intestinal responses in DNA primed – orally boosted pigs were independent of the F4R status, suggesting an increased responsiveness of the parenterally primed animals to oral immunization. In contrast to other studies in mice and pigs demonstrating that vitD₃ enhances IgG but also IgA responses in the inductive sites of the GALT following parenteral immunization (Enioutina *et al.*, 1999, 2000; Van der Stede *et al.*, 2004), addition of vitD₃ to the FaeG DNA vaccine failed to prime for the intestinal mucosal IgA response that is desired to protect piglets against an F4⁺ ETEC infection.

In a second experiment, we evaluated the induction of cellular responses following ID DNA vaccination. A clear FaeG-specific lymphocyte proliferation could only consistently be observed in the presence of the LT vectors. A strong positive influence of these vectors on the antigen-specific cellular response has previously been described (Arrington *et al.*, 2002; Melkebeek *et al.*, 2007). This might result from a higher phenotypic and functional

maturation of APC (Bagley *et al.*, 2002; Martin *et al.*, 2002), resulting in a better antigen-presentation in the presence of LT and thus in a higher number of FaeG-specific memory lymphocytes and/or from a different subset of T(h)-cells and/or cytokine-profile after immunization. For the latter, evidence was obtained by analyzing cytokine mRNA profiles in the Cerv LN.

Pro-inflammatory cytokines were already weakly induced by the injection on itself (PBS group) and even slightly more by the DNA vector, with only the IL-6 mRNA expression being further up regulated by the FaeG expression. FaeG expression also resulted in an increased TGF- β mRNA expression, especially when an adjuvant was added. TGF- β has been suggested to be involved in the mucosal homing of immunocompetent cells (Enioutina *et al.*, 1999) and is known to play a role in the isotype switching towards IgA (Lebman *et al.*, 1990; Iwasato *et al.*, 1994). For the expression of IL-10 (Th2-like) and IFN- γ (Th1-like) mRNA, a high variability could be observed among the animals. Furthermore, in line with other studies in pigs (Suradhat *et al.*, 2003; Reddy *et al.*, 2000), expression of IL-4 (Th2-like) and IL-2, could not be detected. It has been speculated that in pigs other molecules may substitute functionally for classical murine cytokines, like IL-4 and perhaps IL-2 (Reddy *et al.*, 2000). IL-4 function might be substituted by IL-13 (Reddy *et al.*, 2000), which has not been measured here. Consequently, we were not able to attribute a typical Th-cytokine profile to FaeG DNA vaccination and/or the LT vectors and vitamin D₃. What we did observe was a suppression of the FaeG-specific lymphocyte proliferation with increased IL-10 mRNA levels. This is in line with studies in man and cattle demonstrating that IL-10 inhibits Th1- and Th2-cell proliferation in these species (Del Prete *et al.*, 1993; Brown *et al.*, 1994). Moreover, although IL-10 mRNA expression was highly variable, the absence of a clear IL-10 response in the LT group might at least have contributed to the strong FaeG-specific proliferation observed in the presence of the LT vectors. Furthermore, IL-10 synergizes with TGF- β to increase the efficiency of IgA switching (Defrance *et al.*, 1992), while IL-6, together with IL-5 acts to enhance the IgA secretion (Snoeck *et al.*, 2006b). So, although no serum IgA response could be observed in the present study (as discussed above), suppression of both IL-6 and IL-10 mRNA expression by the LT vectors might explain the suppressed serum IgA response in our previous study (Melkebeek *et al.*, 2007).

In conclusion, even after the oral F4 boost, priming of an intestinal mucosal antibody response remained weak, varied among the pigs and seemed to require co-administration of the LT vectors or vitD₃. Moreover, even in presence of these adjuvants, we failed to demonstrate the secretory IgA response that is needed to be protective against ETEC.

Therefore, further research should be directed towards enhancing the IgA response following DNA vaccination.

Acknowledgements

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PART IV

GENERAL

DISCUSSION

GENERAL DISCUSSION

It was previously shown that oral immunization of weaned F4-receptor positive (F4R⁺) pigs with F4 fimbriae results in an antigen-specific secretory IgA (sIgA) response at the intestinal mucosa, protecting these pigs against a subsequent F4⁺ ETEC challenge (Van den Broeck *et al.*, 1999a). However to prevent postweaning diarrhea (PWD), the immune system should already be primed during the suckling period. Most suckling pigs have maternal antibodies against F4 fimbriae, hampering the use of F4 fimbriae as a vaccine. As DNA vaccines produce antigen endogenously, maternal antibodies can not interfere with the induction of cellular immunity by these vaccines. Therefore, we suggested that priming with a DNA vaccine encoding the adhesive subunit of F4, the FaeG, could be an interesting approach to obtain protective immunity against PWD.

The best route to induce an intestinal mucosal immune response is the oral route. However, oral administration of ‘naked’ DNA vaccines is difficult due to the harsh environment of the gastrointestinal tract. Parenteral vaccination seems more appropriate, but tends to stimulate the systemic rather than the mucosal immune system. In efforts to improve mucosal immune responses to naked DNA vaccines, heterologous prime – boost strategies combining parenteral DNA vaccination with mucosal delivery of live attenuated microorganisms or protein-based vaccines have been proposed. Furthermore, studies in mice (Enioutina *et al.*, 1999; 2000; Glenn *et al.*, 1998) and pigs (Van der Stede *et al.*, 2001; 2004) demonstrated that co-administration of immunomodulators, such as 1 α ,25-dihydroxyvitamin D₃ (vitD₃), cholera toxin (CT) or the *E. coli* thermolabile enterotoxin (LT), to systemic protein vaccines not only improved systemic antibody responses but also resulted in the induction of an intestinal mucosal IgA response. In mice, this was at least partly due to an altered migration of antigen-carrying APC, resulting in their localization at intestinal immune induction sites where mucosal antigen-specific IgA and IgG ASC are induced.

The aim of the present thesis was to determine if parenteral DNA vaccination in the presence or absence of immunomodulating adjuvants could reach the intestinal mucosal immune system, either on itself or in a heterologous parenteral DNA prime – oral F4 protein boost protocol. As our previous pcDNA1/*faeG*19 DNA vaccine induced only marginal immune responses following parenteral immunization of pigs, the immunogenicity of our FaeG DNA vaccine needed to be improved first.

Optimization of the FaeG DNA vaccine

Systemic humoral responses: IgG and IgM

When intramuscular (IM) needle injection and intradermal (ID) gene gun delivery of our previous pcDNA1/faeG19 DNA vaccine were compared in pigs, the IM route appeared to be superior with respect to priming of humoral responses (Verfaillie *et al.*, 2004). The same was observed by Deml and colleagues in mice (2001), whereas opposite results are obtained in most other studies comparing both techniques in mice (Stasikova *et al.*, 2003), monkeys (Lodmell *et al.*, 2001) and pigs (Barfoed *et al.*, 2004). These contradicting findings suggest that besides the delivery route and method, the encoded antigen also contributes to the outcome of the immunization. Besides in the inoculation route, gene gun delivery additionally differs from IM needle injection in the lower amount of plasmid DNA and consequently of CpG motifs applied, and in an increased efficacy of cell transfection which may both influence strength and type of immune response. Indeed, gene gun immunization generally induces a Th2-like response, whereas a predominantly Th1-like response is induced by IM as well as ID needle injection in mice (Barry and Johnston, 1997; Feltquate *et al.*, 1997). This has been ascribed to the delivery of different danger signals to APC following each immunization route. While danger signals of IM DNA vaccination only result from CpG motifs and are Th1-like, an additional Th-1 like danger signal is delivered by the physical trauma of ID injection, whereas the process of gold particle bombardment, which only gives a weak trauma, delivers the most important danger signal following gene gun immunization, resulting in a Th2-like response (Liu *et al.*, 2005). Despite these observations in mice, we again observed a better priming of humoral responses in pigs by the IM route when IM needle injection of pWRGFaeG was compared to ID needle injection as shown in chapter 2.

However when GM-CSF DNA was administered one week before the first ID DNA vaccination, priming of the serum IgG response was enhanced and became superior to IM immunization (chapter 2). Therefore, we opted for ID DNA vaccination with GM-CSF pre-treatment in all our following studies. GM-CSF has received considerable attention as an adjuvant for DNA vaccines given its role not only as a growth and differentiation factor for APC but also as a chemo-attractant for these cells. In line with studies in mice (Bowne *et al.*, 1999; Haddad *et al.*, 2000), a further analysis of the adjuvant effect of the GM-CSF gene showed an expansion of APC at the immunization site, but also at the immune induction site until day 4 after FaeG DNA vaccination (chapter 3). These findings suggest not only that

direct transfection of APC and/or the uptake of the expressed antigen by these cells might be increased, but also that the immune response can be induced earlier when GM-CSF DNA is delivered prior to DNA vaccination. Regarding the latter, it would be interesting to know whether the adjuvant effect remains when the GM-CSF gene is co-delivered with the DNA vaccine. Several studies in mice (Yoon *et al.*, 2006) as well as in pigs (Cedillo-Baron *et al.*, 2001; Somasundaram *et al.*, 1999; Dufour *et al.*, 2000) have indeed reported an improved potency of DNA vaccines when the GM-CSF gene was co-administered. Nevertheless, an enhanced density of APC at the immunization site will only be achieved from a few days after the immunization in presence of the GM-CSF gene onwards, which can result in an enhanced uptake of the expressed antigen by these cells but is unlikely to improve their direct transfection. Studies comparing both methods are necessary to determine whether this has a large impact on the induction of systemic responses. However, since priming of mucosal responses following parenteral immunization is believed to result from the migration of antigen-pulsed APC to mucosal inductive sites (Enioutina *et al.*, 1999, 2000, 2007) (as further will be discussed), we choose to deliver the GM-CSF gene in advance to obtain both effects in order to target as much APC as possible.

As DNA vaccination requires the *in vivo* expression of the encoded antigen, the level of protein expression has a direct impact on the magnitude of the induced response (Montgomery *et al.*, 1993). Therefore, we aimed to optimize the FaeG expression by (1) inserting a 'Kozak' consensus sequence for eukaryotic translation initiation into pcDNA1/faeG19 (Kozak, 1987) (pcDNA1/faeGK), (2) using the pWRG7079 vector containing intron A which acts as an enhancer of the CMV IE promoter (Chapman *et al.*, 1991; Wang *et al.*, 2006), the BGH polyadenylation site (polyA) which is more favourable for expression than the SV40 polyA used by pcDNA1 (Montgomery *et al.*, 1993; Norman *et al.*, 1997) and the human tissue plasminogen activator signal sequence to allow extracellular secretion of the encoded antigen (pWRGFaeG) and (3) adjusting the codon-usage of the bacterial *faeG* to that of highly expressed porcine genes (pWRGFaeGopt) (chapter 4). As expected, the *in vitro* FaeG expression increased in the order pcDNA1/faeG19, pcDNA1/faeGK, pWRGFaeG, pWRGFaeGopt. Following ID immunization with pcDNA1/faeG19, pWRGFaeG and pWRGFaeGopt, an F4-specific serum IgG response could be observed of which not only the height but also the speed of onset was improved in line with increasing FaeG expression, already resulting in an F4-specific antibody response two weeks after the first immunization with pWRGFaeGopt. Rapid induction of an F4-specific immune response might be of critical importance as an effective vaccine against PWD should

already induce protective immunity at weaning, when pigs are approximately 4 weeks old. Besides a better antigen expression, the presence of an extracellular secretion signal in the pWRG7079 vector might be beneficial for the induction of humoral responses as it increases the amount of circulating antigen available for Ig receptors and activation of B cells. However, it is not clear whether this will be an advantage in the presence of maternal antibodies. Maternal antibodies will bind the antigen released by transfected cells, thereby masking B cell epitopes and hindering the induction of humoral responses in a similar manner as following conventional vaccination (reviewed by Bot and Bona, 2002). Therefore, B cell responses are unlikely to be induced in the presence of high titers of maternal antibodies. Furthermore, uptake of antigen-maternal antibody complexes by APC via the FcR γ receptor generally doesn't lead to efficient antigen presentation following conventional vaccination in young animals. However, DNA vaccines might activate these APC via CpG motifs to effectively process immune complexes and present class I- and II-restricted epitopes in an immunogenic form (Bot and Bona, 2002). In this regard, antigen-secretion would improve the induction of T cell responses and therefore priming of memory responses. However, studies comparing the efficacy of DNA vaccines encoding secreted and non-secreted antigens in presence of maternal antibodies remain to be done to prove this hypothesis.

Addition of plasmid vectors encoding the A and B subunits of the *E. coli* thermolabile enterotoxin (LT) (together called the LT vectors), and to a lesser extend of 1 α ,25-dihydroxyvitamin D₃ (vitD₃) further improved systemic F4-specific humoral responses, as evidenced by an increased serum IgG response (chapter 4 and chapter 5) and an enhanced isotype switching from IgM to IgG in the spleen after an oral F4 boost (chapter 5).

Although a clear IgM response could be observed in the local draining cervical lymph node (Cerv LN) as well as in the spleen following DNA vaccination (chapter 5), an F4-specific serum IgM response could not be observed in any of our studies. This is likely due to the high cut off value in the IgM ELISA. An IgM background is often observed when young animals are tested and is probably non-specific as even in gnotobiotic piglets, cut off values near 0.4 could be observed using an F4-specific serum IgM ELISA (unpublished data) and a low background of F4-specific IgM ASC is seen in lymphoid tissues of non-immunized animals (Van den Broeck *et al.*, 1999b; Verdonck *et al.*, 2002). However compared to the situation in serum, we observe less problems due to background when looking at IgM responses in target lymphoid tissues as the antibody response is localized in these tissues shortly after immunization, often resulting in antigen-specific IgM ASC numbers that clearly exceed the background. Furthermore, other tissues like the bone marrow and other peripheral lymph

nodes might have contributed to the observed serum antibody response. We did not examine these tissues, which might explain the absence of a correlation between the situation in serum and that in lymphoid tissues.

Systemic cellular responses

Comparing IM and ID needle injection of the FaeG DNA vaccine revealed that the ID route is superior to induce cell-mediated immunity against F4 (chapter 2). The same was observed by Verfaillie and colleagues (2004) when the IM injection of pcDNA1/faeG19 was compared to gene gun delivery and might reflect the better immune surveillance function of the skin compared to muscle. Skin contains many specialized antigen-presenting cells (APCs) [e.g. Langerhans cells (LC) in the epidermis, and dendritic cells (DC) and macrophages in the dermis] which have been shown to be essential for the induction of cellular immune responses after DNA vaccination (Casares *et al.*, 1997; Fu *et al.*, 1997; Iwasaki *et al.*, 1997), whereas clearly less comparable cells are present in muscles. These APC are involved in T cell priming in at least 3 different ways: they can capture antigen secreted by other transfected cells, resulting in MHC class II-restricted presentation, pick up antigen from transfected cells by an as yet ill-defined mechanism, resulting in MHC class I-restricted “cross”-presentation or they can be transfected themselves, resulting in MHC class I presentation of plasmid encoded antigens (Takashima and Morita, 1999). That enhancing the number of APC at the immunization site by delivering the GM-CSF gene prior to DNA vaccination further improves the cellular response supports this theory (chapter 2).

An improvement in induction of F4-specific cellular responses by expression optimization or co-administration of vitD₃ could not be demonstrated as a clear FaeG-specific lymphocyte proliferation was only consistently observed in the presence of the LT vectors as can be seen in chapters 4 and 5. The absence of a positive effect of vitD₃ on proliferation is in agreement with the study in pigs evaluating the influence of vitD₃ on IM F4 protein immunization (Van der Stede *et al.*, 2003). Moreover *in vitro*, a suppressive effect on T cell proliferation has been described for vitD₃ (van Etten and Mathieu, 2005; Bhalla *et al.*, 1986).

The positive effect of the LT vectors on the induction of cellular responses by DNA vaccination was also observed by Arrington *et al* (2002) in mice. They showed that the adjuvant effects were caused by the assembled LT holotoxin and to a lesser extend by the LTB subunit and not by CpG motifs in these vectors since the LTA vector on itself, but also the LTB vector on itself when the signal sequence was deleted, had no adjuvant effects.

However, Arrington vaccinated via the gene gun whereas we used ID needle injection to deliver the LT vectors. As CpG motifs exert their immunostimulatory potential by a pathway involving receptor-mediated endocytosis of the CpG-containing DNA into immune cells (macrophages, DC, and B cells), gene gun immunization may possibly bypass this signaling mechanism because of the direct intracellular deposition of the plasmid (Haynes, 1999). Therefore, we can not exclude a possible CpG effect in our study.

In mice, the enhanced cellular immunity using the LT vectors was accompanied by a Th1-modulating effect (Arrington *et al.*, 2002). Conversely, vitD₃ has been shown to suppresses production of Th1 cytokines (van Etten and Mathieu, 2005; Bhalla *et al.*, 1986; Reichel *et al.*, 1987) and to favor the production of Th2 cytokines such as IL-4 and IL-10 (Daynes and Araneo, 1994; Daynes *et al.*, 1996; Cantorna *et al.*, 1998; Adorini *et al.*, 2001) as well as of the Th3 cytokine TGF- β (Fukaura *et al.*, 1996; Weinreich *et al.*, 1999). To determine the cytokine profile in pigs, expression of IL-1 α , IL-6, TNF- α (pro-inflammatory cytokines), IL-2, IFN- γ (Th1-like cytokines), IL-4, IL-10 (Th2-like cytokines) and TGF- β (Th3-like cytokine) mRNA was evaluated in the Cerv LN (chapter 5). We were not able to attribute a typical Th-cytokine profile to FaeG DNA vaccination and/or the LT vectors and vitD₃ as a high variability could be observed in IL-10 (Th2-like) and IFN- γ (Th1-like) mRNA expression, whereas expression of IL-4 (Th2-like) and IL-2 (Th1-like) could not be detected. The absence of a detectable IL-2 and IL-4 mRNA expression is in line with other studies in pigs (Suradhat *et al.*, 2003; Reddy *et al.*, 2000). It has been speculated that other cytokines might substitute functionally for these classical cytokines in pigs (Reddy *et al.*, 2000). IL-13 may substitute IL-4 in steering Th2 differentiation (Reddy *et al.*, 2000). Indeed, IL-13 shares many functional properties with IL-4, including stimulation of murine B cells, inhibition of pro-inflammatory cytokine production, induction of IL-1 receptor antagonist by monocyte/macrophages and the regulation of isotype class switching in B cells to IgE synthesis (De Waal Malefyt *et al.*, 1993; Cocks *et al.*, 1993; Defrance *et al.*, 1994). These shared functions are due to a common receptor chain (Wills-Karp, 2004). For IL-2, a substitute has not yet been proposed.

We did observe an increased IL-6 and TGF- β mRNA expression by the FaeG DNA vaccination. Both the LT vectors and vitD₃ further enhance the TGF- β mRNA expression, whereas the LT vectors also seem to suppress IL-6 mRNA expression.

Although IL-10 mRNA expression was highly variable, increased IL-10 mRNA levels were consistently accompanied by a suppression of FaeG-specific lymphocyte proliferation. This is in agreement with studies in man and cattle demonstrating inhibited Th1- and Th2-cell

proliferation by IL-10 (Del Prete *et al.*, 1993; Brown *et al.*, 1994). Moreover, the absence of a clear IL-10 response in the LT group might at least have contributed to the strong FaeG-specific proliferation observed in this group.

Priming of a serum IgA response

When the IM and the ID route in the absence of GM-CSF were compared, only IM injection resulted in a secondary serum IgA response following an IM F4 protein boost. However, when the GM-CSF gene was given 7 days prior to the IM DNA vaccination, than this immunization resulted in an even more efficient priming for IgA (chapter 2). Further enhancement of the IgA response was obtained by vector-codon-optimization of the DNA vaccine resulting in a vaccine-induced IgA response in serum (chapter 4) or Cerv LN (chapter 5).

The better IgA response observed in chapter 4 than in chapter 5 might result from differences in environmental factors encountered by the pigs, or alternatively from an unnoticed minor contact with F4. Indeed, it has been described that intestinal priming can result in an IgA response following parenteral immunization (Svennerholm *et al.*, 1980). Although farms were carefully selected, litters were tested to be F4-seronegative and pigs were orally treated with colistine to prevent infection during the weaning period, a risk for such a contact can not absolutely be excluded.

Although parenteral immunization preferentially induces an IgG response, induction of a systemic IgA response was also seen in earlier studies in pigs (Vanderstede *et al.*, 2002, Vanderpooten *et al.*, 1997) and was more efficient by immunizing in the neck compared to the back. This might be explained by the fact that the dorsal superficial cervical LN (draining the neck region) is connected to lymph nodes draining the tonsils and the nasal mucosa, which might be more beneficial for IgA induction (Vanderpooten *et al.*, 1997).

When LT or vitD₃ are added to parenteral protein vaccines, an IgA-modulating effect can be observed (Daynes and Araneo, 1994; Daynes *et al.*, 1996; Enioutina *et al.*, 1999, 2000; Yu *et al.*, 2002; Van der Stede *et al.*, 2001, 2004). However, adding the LT vectors to the optimized DNA vaccine abolished the serum IgA response (chapter 4), whereas no increase in IgA response could be observed when vitD₃ was added (chapter 5). It is possible that either GM-CSF pre-treatment, the presence of CpG motifs and/or by the slow and continuous expression of low antigen/LT doses following DNA vaccination promotes different APC or T-lymphocyte functions, neutralizing the effect of vitD₃ and the expressed LT. Furthermore, the nature of the expressed antigen might have influenced the immunomodulating effect.

Indeed, although vitD₃ enhanced the IgA response to human serum albumin (Van der Stede *et al.*, 2001, 2004), it did not enhance the IgA response to F4 fimbriae in pigs (Van der Stede *et al.*, 2003). In addition, as IL-10 is involved in IgA isotype switching (Defrance *et al.*, 1992) and IL-6 acts to enhance the IgA secretion (Snoeck *et al.*, 2006b), suppression of these cytokines by the LT vectors might explain the suppressed IgA response by these vectors.

Priming of an intestinal mucosal immune response by parenteral DNA vaccination

VitD₃ and LT have been demonstrated to promote the induction of mucosal immune responses following parenteral delivery of protein antigens (Enioutina *et al.*, 1999, 2000; Glenn *et al.*, 1998; Van der Stede *et al.*, 2001, 2003, 2004). This has been ascribed to their ability to alter the migratory properties of antigen-loaded DC, allowing them to bypass sequestration in the local draining lymph node and to traffic to the Peyer's patches, where antigen-specific IgA and IgG ASC are induced (Enioutina *et al.*, 2000). However, in the presence of the LT vectors or vitD₃, we could not consistently demonstrate an intestinal mucosal response following DNA vaccination (chapter 5). Similarly, even though the group of Daynes observed mucosal responses in mice that were quantitatively and qualitatively similar to those induced by oral immunization in the presence of cholera toxin as adjuvant when they included vitD₃ in a parenteral protein vaccine, they failed to observe mucosal antibodies following parenteral DNA vaccination in the presence of vitD₃ (Kriesel *et al.*, 1996). As already suggested for the absence of a serum IgA response, it is conceivable that either GM-CSF pre-treatment, the presence of CpG motifs and/or the slow and continuous production of low antigen amounts following DNA vaccination might have counteracted the immunomodulating effect of both adjuvants. Indeed, while GM-CSF and CpG motifs have been described to activate DC, vitD₃ tends to delay full DC maturation (Berer *et al.*, 2000; Griffin *et al.*, 2000, 2003). Whether any relationship exists between the mechanisms underlying the ability of LT or vitD₃ to promote the generation of mucosal responses is uncertain. For the mucosal adjuvant properties of LT, the ability to elevate cAMP levels seems to be important. Enioutina *et al.* (2000) hypothesized that this might be due to the capacity of cAMP to stimulate the expression of 1,α-hydroxylase, which converts the normal circulating 25(OH)D₃ into the active 1,25(OH)₂D₃ form, referred to as vitD₃ in this thesis. Although cAMP activity could be demonstrated following *in vitro* transfection of Caco2 cells with the LT vectors (Arrington *et al.*, 2002), it is likely that formation of a functional AB₅ LT holotoxin and thus elevation of cAMP levels is less efficient *in vivo*, explaining the absence of

a mucosal adjuvant effect by these vectors. In addition, the slow and continuous production of low amounts of the LTA and LTB subunits and/or the assembled LT might be less effective than the short-term presence of the larger amount of LT administered with protein antigens. However, significant mucosal antibody responses have been observed in rabbits and monkeys following IM DNA vaccination when the encoded antigen was fused to CTLA-4 (Jia *et al.*, 2006). CTLA-4 binds the B7 (CD80/86) costimulatory molecules on the surface of APC, thereby targeting the fused antigen to these cells. Whether any relationship exists between the mechanisms underlying the ability of CTLA-4, vitD₃ and cAMP to promote priming of mucosal responses is unclear, but the function of CTLA-4 additionally highlights the key role of APC in the priming of mucosal responses following parenteral immunization. As combining a CTLA-4 fusion to GM-CSF pre-treatment could further enhance APC targeting in our model, this might be an interesting approach to obtain mucosal priming. Furthermore, the ability of DNA-transfected APC to bypass the local draining lymph node has been demonstrated (La Cava *et al.*, 2000). In that study, ID DNA vaccination in the presence of an inflammatory adjuvant resulted in APC-mediated transport of DNA beyond the draining LN to distant sites such as the spleen. Moreover, when a second site of inflammation was induced, plasmid DNA accumulated at this distal site. These data suggest that coupling parenteral DNA vaccination and an intestinal inflammatory stimulus might be more effective to target the immune response to the intestinal mucosa.

Another strategy that has been applied successfully to improve the induction of mucosal responses following parenteral DNA vaccination includes the delivery of a mucosal boost with live attenuated microorganisms or protein-based vaccines (Weeratna *et al.*, 2000; Lasaro *et al.*, 1999, 2004). In the present thesis, oral F4 administration boosted the serum IgG, but not the serum IgA response that was induced by pWRGFaeGopt (chapter 4). Although the IgG response remained mainly localized in the systemic lymphoid tissues, a weak mucosal IgG response could be observed (chapter 4 and chapter 5). This mucosal response varied considerably among the pigs whereas low numbers of IgG ASC were more consistently detected in the Mes LN of most DNA primed pigs, especially if the LT vectors or vitD₃ were added. In contrast to the situation in naïve (unprimed) pigs where oral F4 administration to F4R⁻ pigs fails to induce a mucosal response and is less efficient to prime a systemic response than in F4R⁺ pigs (Van den Broeck *et al.*, 1999a), induction of these systemic and mucosal IgG responses was independent of the F4R status. This is consistent with other reports demonstrating the induction of immune responses following oral immunization of parenterally primed, but not of naïve animals (Lauterslager *et al.*, 2001, 2003), indicating an

increased responsiveness of the parenterally primed animals to oral immunization. Possible explanations for this increased responsiveness might include an improved uptake of the antigen through the gut, an altered antigen-processing favouring immunoresponsiveness rather than oral tolerance and an enhanced capability of the primed immune system to react to small quantities of antigen entering the body. Nevertheless, only strongly F4R⁺ pigs showed high numbers of IgG ASC in their LP and low levels of IgG antibodies in their intestinal contents.

Following the oral boost, intestinal FaeG-specific IgA and IgM responses were variable and an improvement by DNA priming could only be observed for a few pigs, mostly when the LT vectors or vitD₃ were added (chapter 4 and chapter 5).

Although sIgA generally provides the primary defence against intestinal pathogens, parenteral priming with pWRGFaeGopt followed by an oral F4 protein boost marginally but significantly reduced the faecal F4⁺ *E. coli* excretion, whereas co-administration of the LT vectors additionally reduced the duration of faecal excretion following an F4⁺ ETEC challenge one week after the oral F4 boost (chapter 4). This partial protection might be attributed to the IgG response. Indeed, a study by Yu and colleagues (2002) demonstrated protection of mice against an oral LT challenge by passive infusion of serum derived anti-LT IgG. IgG can be transported towards the intestinal lumen via the bidirectional IgG transporting neonatal Fc receptor (FcRn) (Dickinson *et al.*, 1999), which might explain the presence of F4-specific IgG in the intestinal contents of some pigs immunized by the heterologous protocol. Expression of this FcRn receptor has been demonstrated on the intestinal epithelium of both young and adult humans and pigs (Israel *et al.*, 1997; Stirling *et al.*, 2005). Once in the intestinal lumen, IgG could contribute to protection. In one study, oral administration of milk derived anti-CFA/I IgG protected human volunteers against a CFA/I⁺ ETEC infection (Freedman *et al.*, 1998). However in general, the ability of pathogen-specific IgG to protect against infection at the mucosal surfaces is rather limited. It can neutralize bacteria, mediate antibody-dependent cellular cytotoxicity and activate complement, but unlike sIgA, IgG is poorly equipped to prevent bacterial attachment to the mucosal surface or to trap bacteria in mucus (Robert-Guroff, 2000).

Although several studies have demonstrated the successful induction of mucosal immunity by parenteral DNA vaccination followed by a heterologous mucosal boost, virtually all these studies were done in mice (Weeratna *et al.*, 2000; Lasaro *et al.*, 1999, 2004). A possible reason for the better results obtained in those studies may be the higher dose of DNA used relative to the size of the animal's intestine. Indeed, although the total intestinal mass of the

mouse is much smaller than that of a pig, the dose of DNA they used to prime mucosal responses was only 5 times lower than the dose we used (Lasaro *et al.*, 1999, 2004). However, it remains to be determined whether using larger DNA doses would allow more successful priming of the intestinal mucosal immune system in our pig model. In agreement with our results, Yuan *et al.* (2005) also failed to protect pigs against rotavirus infections by intramuscular (IM) priming with DNA followed by an oral boost with attenuated human rotavirus (AttHRV). However, they did observe protection when the oral AttHRV was given to prime and the IM DNA vaccine to boost the immune response in pigs. This protection was associated with a higher serum IgA and a higher intestinal IgA and IgG response. Similarly, Eo and colleagues (2001) observed excellent mucosal responses by priming IN with a recombinant vaccinia virus vector (rvac) and boosting systemically with a DNA vaccine, but not by systemic DNA priming and mucosal boosting with rvac or by systemic rvac priming and mucosal boosting with the DNA vaccine. These data indicate that the anatomic site where initial APC-T cell interactions occur determines the success to induce a mucosal response. However, as stated before, mucosal delivery of ‘naked’ DNA vaccines is not ideal due to the harsh enzymatic environment and the limited transport across the mucosal epithelium. Nevertheless, in rodents, DNA vaccines have been proven to be capable of inducing protective mucosal IgA responses when strategies are used to overcome these barriers. These strategies include the use of formulations protecting the DNA from degradation and/or targeting the DNA to mucosal inductive sites (Wu *et al.*, 2001; O’Hagan *et al.*, 2004; Alpar *et al.*, 2005; Wang *et al.*, 2005) and the use of live bacterial carriers to deliver the DNA vaccine to mucosa-associated lymphoid tissues (Schoen *et al.*, 2004) and are discussed in detail in chapter 1. However, as these studies have almost exclusively been performed in rodents, their potential in large animal models such as the pig remains to be investigated.

Main conclusions and future perspective

DNA vaccines have received considerable attention after the initial demonstration in the early 90’s that unformulated plasmid DNA could result in *in vivo* expression of the encoded protein after IM injection (Wolff *et al.*, 1990) and induce both humoral and cellular responses against human pathogens (Ulmer *et al.*, 1993; Fynan *et al.*, 1993; Wang *et al.*, 1993; Davis *et al.*, 1994). However although these initial vaccines generally worked well in mice, they were often less immunogenic in large animals and humans. Since then, new formulations, new adjuvants, new delivery methods and more sophisticated constructs have been developed to

improve the potency of these vaccines (Laddy and Weiner, 2006; Liu *et al.*, 2006; Leitner *et al.*, 1999). At present, DNA vaccines finally start to gain a position on the market. However, future challenge remains to make these vaccines more potent and cheaper to manufacture before they can be routinely used.

In this thesis, we demonstrated that the immunogenicity of the parenteral FaeG DNA vaccine in pigs could be greatly improved by determining the optimal delivery route, pre-treatment of the immunization site with GM-CSF DNA and optimizing the FaeG expression. Nevertheless, even in the presence of immunomodulators and/or an oral protein boost, parenteral immunization with this vaccine induced mainly a systemic IgG response, failing to prevent F4⁺ *E. coli* excretion upon challenge. To induce the sIgA response that is needed to prevent postweaning diarrhoea by DNA vaccination, strategies targeting the intestinal mucosa might prove to be more effective. Although oral DNA vaccination is still in its infancy, the use of live bacterial carriers to deliver the DNA vaccine to the gut-associated lymphoid tissues (GALT) (Schoen *et al.*, 2004) and formulations to protect the DNA against degradation and to target it to mucosal inductive sites have had some success in rodents. Regarding the latter, PLG microparticles smaller than 10 µm in diameter have been used as these are actively taken up by M cells of the Peyer's patches and by APC and thus appear able to facilitate the presentation of DNA to the intestinal mucosal immune system (Chen *et al.*, 1998; Herrmann *et al.*, 1999; O'Hagan *et al.*, 2004). However, it seems unlikely that uptake of these microparticles into the GALT is sufficient to allow the successful development of oral vaccines in large animals (O'Hagan *et al.*, 2004). Alternatively, the bioadhesive chitosan nanoparticles have successfully been used for oral delivery of DNA in mice (Chew *et al.*, 2003). These are also taken up by M cells (van der Lubben *et al.*, 2001, 2002) but additionally interact with mucin (Lehr *et al.*, 1992), providing an intimate contact with the mucosal membrane and promoting more efficient uptake. However, their potential in large animals remains to be tested. In addition, coating of microparticles with M cell ligands could be an interesting approach to allow more efficient targeting to the GALT. However, in contrast to other species, lectins specific for the apical surface of porcine M cells have not yet been identified. Nevertheless, the $\sigma 1$ protein of reovirus and the invasin of *Yersinia*, both exploiting the membrane protein $\beta 1$ -integrin, apically located on M cells, to gain entry into the host (Maginnis *et al.*, 2006; Clark *et al.*, 1998; Schulte *et al.*, 2000), may be used for selective targeting to M cells. Wang *et al* (2003) and Wu *et al* (2001) have already demonstrated that the reovirus $\sigma 1$ protein can be used for selective targeting of DNA-

polylysine complexes to nasal M cells. However, although the $\sigma 1$ protein also binds Peyer's patch M cells (Amerongen *et al.*, 1994; Clark *et al.*, 1995), it has not been tested in oral delivery systems yet. In addition, in rodents and rabbits, Peyer's patches M cells selectively bind and endocytose sIgA (Mantis *et al.*, 2002). This receptor may be conserved among species and may also be exploited for selective targeting to M cells. Zhou *et al* (1995) and Velez *et al* (1997) have already demonstrated that selective targeting to M cells can be achieved by coating carrier liposomes with IgA. Consequently, oral immunization of suckling pigs with our optimized FaeG DNA vaccine using a micro- or nanoparticle system coated with one of these M cell ligands might be promising.

SUMMARY

Enterotoxigenic *E. coli* (ETEC) that express F4 (K88) fimbriae are an important cause of disease, weight loss and mortality in newly weaned piglets. The F4 fimbriae are long polymeric appendages mainly composed of several hundreds identical FaeG subunits. They enable the bacteria to colonize the small intestine and to produce enterotoxins resulting in diarrhoea. In our laboratory it has been demonstrated that oral administration of purified F4 fimbriae to weaned piglets results in an antigen-specific secretory IgA (sIgA) response at the intestinal mucosa, protecting the piglets against a subsequent F4⁺ ETEC challenge. However to prevent postweaning diarrhoea (PWD), the immune system should already be primed during the suckling period. Most suckling pigs have maternal antibodies against the F4 fimbriae, hampering their use as a vaccine.

As DNA vaccines produce their encoded antigen endogenously, maternal antibodies won't interfere with the induction of cellular immunity by these vaccines. Therefore, we suggested that priming with a DNA vaccine encoding the FaeG could be an interesting approach to obtain protective immunity against PWD.

The best route to induce an intestinal mucosal immune response is the oral route. However, oral DNA vaccination has to overcome several problems to be effective. These problems include depurination by low pH in the stomach, enzymatic degradation by DNases in the gut, crossing the mucus layer and transport through the epithelial barrier to reach the intestinal mucosal inductive sites. Parenteral DNA vaccination is easier, but tends to stimulate a systemic rather than a mucosal IgA response. However, studies in mice and pigs demonstrated that co-administration of immunomodulators, such as 1 α ,25-dihydroxyvitamin D₃ (vitD₃), cholera toxin or the *E. coli* thermolabile enterotoxin (LT), with parenteral protein vaccines not only improved systemic antibody responses but also induced an intestinal mucosal IgA response. These immunomodulators alter the migratory properties of antigen-pulsed antigen-presenting cells (APC), resulting in their preferential localization at the Peyer's patches where antigen-specific mucosal responses are initiated.

In the present thesis, we questioned if parenteral FaeG DNA vaccination could prime an intestinal mucosal immune response when immunomodulating adjuvants and/or a mucosal F4 boost were included. The FaeG DNA vaccine previously developed at our lab induced marginal immune responses following parenteral immunization of pigs. In order to enlarge the chance for successful mucosal priming, several strategies were applied to enhance the immunogenicity of our DNA vaccine. Furthermore, given the critical role of APC in the

induction of mucosal immunity by parenteral immunization, granulocyte-macrophage colony-stimulating factor (GM-CSF), a well known chemo-attractant of APC was included in our vaccination strategy.

Chapter 1 reviews the present knowledge on DNA vaccines, with a focus on the potential of these vaccines in pigs. In a first part, the history of DNA vaccines, their principle, the mechanism by which they induce immune responses and their advantages compared to conventional vaccines are described. An important disadvantage is that DNA vaccines are mostly less immunogenic in large animals and humans than in mice. Therefore, in the second part, strategies applied to enhance the efficacy of DNA vaccines in pigs are summarized. In the last part of this chapter, the potential of DNA vaccines to induce a mucosal response is outlined.

Chapters 2 to 5 present the experimental work of this thesis. The following questions were addressed:

- What is the optimal route for parenteral FaeG DNA vaccination, the intradermal (ID) or the intramuscular (IM) route?
- Does pre-treatment with plasmid encoded GM-CSF increase the immunogenicity of our parenteral DNA vaccine and is this associated with a chemo-tactic effect on APC?
- Can the immunogenicity of the parenteral FaeG DNA vaccine further be enhanced by optimizing the FaeG expression?
- Does parenteral priming with the optimized FaeG DNA vaccine in the presence or absence of vitD₃ or plasmids encoding the A and B subunits of LT (together called the LT vectors), either on itself or in a parenteral DNA prime – oral F4 protein boost protocol, prime the intestinal mucosal IgA response needed to protect pigs against PWD?

In chapter 2, IM and ID injection of pWRGFaeG were compared for their capacity to prime systemic cellular and humoral responses in weaned piglets in order to determine the optimal route for parenteral FaeG DNA vaccination. In addition, the adjuvant effect of plasmid DNA encoding the porcine GM-CSF was determined on ID DNA vaccination by delivering it 7 days prior to the first immunization, as plasmid encoded GM-CSF has been shown to attract APC to the injection site in mice with a maximal effect on day 7. The IM route induced better humoral responses since IgG titers were raised earlier and a weak serum IgA response was primed, whereas the ID route was superior in inducing cell-mediated

immunity, as demonstrated by a lymphocyte proliferation assay. ID delivery of the GM-CSF gene one week before the first ID DNA vaccination further enhanced the F4-specific cellular as well as humoral immune responses and even resulted in a more efficient priming of the IgA response than the IM immunization.

Therefore, we further evaluated the adjuvant effect of plasmid DNA encoding the porcine GM-CSF on APC in pigs ([chapter 3](#)). We demonstrated that ID delivery of the GM-CSF gene 7 days prior to ID FaeG DNA vaccination significantly increases the number of epidermal CD1⁺ cells (Langerhans' cells, skin DC) at the immunization site. This was accompanied by an enhanced percentage of APC at the immune induction site until 4 days after FaeG DNA vaccination. These findings suggest not only that direct transfection of APC and/or the uptake of the expressed antigen by these cells might be increased, but also that the immune response can be induced earlier when GM-CSF DNA is delivered prior to DNA vaccination. Furthermore, since priming of mucosal responses following parenteral immunization is believed to result from the migration of antigen-pulsed APC to mucosal inductive sites, increasing APC numbers might improve our chance on successful mucosal priming. Therefore, we opted for ID DNA vaccination with GM-CSF pre-treatment in our following studies.

In [chapter 4](#), it was examined if optimizing the FaeG expression and/or inclusion of the LT vectors could further enhance the immunogenicity of our ID FaeG DNA vaccine. In addition, priming of an F4-specific intestinal mucosal immune response was assessed in an intradermal (ID) DNA prime - oral F4 boost protocol. To optimize the FaeG expression, first, a 'Kozak' consensus sequence for eukaryotic translation initiation was inserted into pcDNA1/*faeG*19 (pcDNA1/*faeGK*). Then, the pcDNA1 vector was replaced by pWRG7079 (pWRGFaeG). As pcDNA1, this vector contains the CMV IE promoter, but in addition it contains the associated intron A, which acts as an enhancer for this promoter and, instead of the SV40 polyadenylation site (polyA) of pcDNA1, it contains the more favourable BGH polyA for transcriptional termination. In addition, pWRG7079 contains the human tissue plasminogen activator (tPA) signal sequence to allow extracellular secretion of the encoded antigen. Finally, the codon-usage of the bacterial *faeG* was adjusted to that of highly expressed porcine genes (pWRGFaeGopt). As expected, the *in vitro* FaeG expression increased in the order pcDNA1/*faeG*19, pcDNA1/*faeGK*, pWRGFaeG, pWRGFaeGopt. Subsequently,

pcDNA1/*faeG19*, pWRGFaeG and pWRGFaeGopt were tested for their immunogenicity in weaned pigs in an ID DNA prime – oral F4 protein boost model. Following ID DNA vaccination, an F4-specific serum IgG response could be observed of which not only the height but also the speed of onset was improved in line with increasing FaeG expression. Consequently, an F4-specific IgG response could already be observed as soon as two weeks after the first immunization with pWRGFaeGopt. Furthermore, only this vector-codon-optimized construct induced a serum IgA response. The LT vectors additionally enhanced the antibody response and were necessary to observe an F4-specific cellular response, but abolished the serum IgA response. These vectors were added because LT has been described to direct the systemic response towards a mucosal IgA response in mice. Here, however, the ID FaeG DNA prime – oral F4 boost immunization resulted mainly in a systemic IgG response. Only few pigs showed an intestinal mucosal immune response with antibodies which were mainly IgG instead of IgA. Normally sIgA provides the primary defence against intestinal pathogens like ETEC. Nevertheless, priming with pWRGFaeGopt significantly reduced the faecal F4⁺ *E. coli* excretion, whereas co-administration of the LT vectors additionally reduced the duration of faecal excretion. These findings suggest a role for IgG in this partial protection.

In [chapter 5](#), we evaluated if addition of vitD₃ to the DNA vaccine could further enhance mucosal priming and/or modulate the antibody response towards IgA in our ID DNA prime (2x) – oral F4 protein boost model. Furthermore, to evaluate the need for the oral F4 boost in the induction of mucosal immunity, the heterologous ID DNA prime (2x)- oral F4 protein boost immunization was compared to a homologous ID pWRGFaeGopt DNA vaccination (3x). A systemic IgG response was induced by both protocols and was higher following the heterologous protocol. Co-administration of the LT vectors, and to a lesser extend of vitD₃, enhanced this response. However, although LT and vitD₃ were shown to direct a systemic response towards a mucosal IgA response when added to parenteral protein vaccines, we could not demonstrate an intestinal mucosal response following parenteral DNA vaccination, regardless of the presence of the LT vectors or vitD₃. Furthermore, vitD₃ did not enhance the systemic IgA response induced by pWRGFaeGopt. After the oral F4 boost, a weak mucosal IgG response could be observed. This mucosal response varied considerably among the pigs whereas low numbers of IgG antibody-secreting cells (ASC) were more consistently detected in the mesenteric lymph nodes of the DNA primed pigs, especially if the LT vectors or vitD₃ were added. However, despite two DNA immunizations in the presence of these adjuvants

and an oral F4 boost, we failed to demonstrate the secretory IgA response needed to be protective against enterotoxigenic *E. coli*. In a second experiment, the induction of cytokine mRNA expression and lymphocyte proliferation were evaluated following ID DNA vaccination in the presence or absence of the LT vectors or vitD₃. A clear FaeG-specific lymphocyte proliferation could only consistently be observed in the presence of the LT vectors. When cytokine mRNA expression was evaluated in the local draining lymph nodes, an increase in IL-6 and TGF- β mRNA expression was observed by pWRGFaeGopt vaccination. Both the LT vectors and vitD₃ further enhance the TGF- β mRNA expression, whereas the LT vectors also seem to suppress IL-6 mRNA expression. Although IL-10 mRNA expression was highly variable, increased IL-10 mRNA levels were consistently accompanied by a suppression of FaeG-specific lymphocyte proliferation. This is in agreement with studies demonstrating inhibited T-helper (Th)1- and Th2-cell proliferation by IL-10 in man and cattle. Moreover, the absence of a clear IL-10 response in the LT group might at least have contributed to the strong FaeG-specific proliferation observed in this group. Furthermore, as IL-10 is involved in IgA isotype switching and IL-6 acts to enhance the IgA secretion, suppression of these cytokines by the LT vectors might explain the suppressed IgA response observed by these vectors in chapter 4.

The final chapter, (chapter 6) represents the general discussion, conclusions and future perspectives with respect to the obtained results. In this thesis, we demonstrated that the immunogenicity of the parenteral FaeG DNA vaccine in pigs could greatly be improved by determining the optimal delivery route, pre-treatment of the immunization site with GM-CSF DNA and optimizing the FaeG expression. Nevertheless, even in the presence of immunomodulators and/or an oral protein boost, parenteral immunization with this vaccine mainly induced a systemic IgG response, failing to prevent F4⁺ *E. coli* excretion upon challenge. To induce the sIgA response that is needed to prevent postweaning diarrhoea by DNA vaccination, the oral route might prove to be more effective. Although oral DNA vaccination is still in its infancy, the use of live bacterial carriers to deliver the DNA vaccine to the gut-associated lymphoid tissues (GALT) and formulations to protect the DNA against degradation and to target it to mucosal inductive sites have been proposed. Especially strategies that selectively target the DNA to M cells might be promising.

SAMENVATTING

Enterotoxigene *Escherichia coli* (ETEC) die F4 (K88) fimbriae tot expressie brengen zijn een belangrijke oorzaak van ziekte, gewichtsverlies en sterfte bij pasgespeende biggen. De F4 fimbriae zijn lange polymeren die voornamelijk opgebouwd zijn uit een honderdtal identieke FaeG subeenheden. Ze laten de bacteriën toe om de dunne darm te koloniseren en enterotoxines te produceren die resulteren in diarree. In ons laboratorium is reeds aangetoond dat orale toediening van gezuiverde F4 fimbriae aan gespeende biggen een F4-specifieke secretorische IgA (sIgA) respons induceert ter hoogte van de intestinale mucosa die de biggen beschermt tegen een F4⁺ ETEC challenge infectie (Van den Broeck *et al.*, 1999a). Om speendiarree (PWD) te kunnen voorkomen dient het immuunsysteem echter reeds “geprimed” te worden tijdens de zoogperiode. De meeste zogende biggen hebben maternale antistoffen tegen de F4 fimbriae die het gebruik van deze fimbriae als vaccin belemmeren.

Aangezien DNA vaccins hun antigeen *de novo* aanmaken binnenin de getransfecteerde gastheercel gaan maternale antistoffen niet interfereren met het induceren van cellulaire immuniteit door deze vaccins. Daarom veronderstellen we dat immunisatie van zogende biggen met een DNA vaccin dat codeert voor het FaeG een interessante aanpak zou kunnen zijn om bescherming te induceren tegen PWD.

De beste route om een intestinale mucosale immuunrespons te induceren is de orale route. Om effectief te kunnen zijn moeten orale DNA vaccins echter eerst verschillende problemen overwinnen. Deze problemen omvatten depurinatie door de lage pH in de maag, enzymatische degradatie door DNasen in de darm, doorheen de mucus laag geraken en doorheen het epitheel getransporteerd worden om de immunologische inductieplaatsen in de darm te kunnen bereiken. Parenterale immunisatie is gemakkelijker maar zal eerder een systemische dan een mucosale IgA respons induceren. Desondanks hebben studies in muizen en biggen aangetoond dat toevoegen van immunomodulators, zoals 1 α ,25-dihydroxyvitamine D₃ (vitD₃), cholera toxine of het *E. coli* thermolabiel enterotoxine (LT) aan parenterale proteïnevaccins niet alleen de systemische antistoffenrespons verbetert maar ook resulteert in een intestinale mucosale IgA respons. Deze immunomodulators wijzigen het migratie patroon van antigeen-getransfecteerde antigeen-presenterende cellen (APC) waardoor deze zich bij voorkeur ter hoogte van de Peyerse platen gaan lokaliseren, waar ze de antigeen-specifieke mucosale immuunrespons gaan initiëren.

In deze thesis hebben we nagegaan of parenterale FaeG DNA vaccinatie een intestinale mucosale immuunrespons kan “primen” indien immunomodulerende adjuvantia en/of een

mucosale F4 “boost” toegevoegd worden. Het FaeG DNA vaccin dat eerder ontwikkeld was in ons labo induceerde marginale immuunresponsen na parenterale immunisatie van biggen. Om de kans op een succesvolle mucosale priming te verhogen werden verschillende strategieën toegepast om de immunogeniciteit van dit DNA vaccin te verhogen. Omwille van de kritische rol van APC in het induceren van een mucosale respons na parenterale immunisatie werd bovendien ook de granulocyt-macrofaag kolonie-stimulerende factor (GM-CSF), een welgekend chemo-attractant van APC ingevoegd in onze vaccinatiestrategie.

Hoofdstuk 1 geeft een overzicht van de huidige kennis over DNA vaccins, met de nadruk op het potentieel van deze vaccins bij varkens. In het eerste deel worden de geschiedenis van DNA vaccins, hun principe, de manier waarop ze een immuunrespons induceren en hun voor- en nadelen ten opzichte van conventionele vaccins beschreven. Een belangrijk nadeel is dat DNA vaccins meestal minder immunogeen zijn bij grote diersoorten en de mens dan bij muizen. Daarom wordt in het tweede deel een samenvatting gegeven van de strategieën die reeds toegepast zijn om de doeltreffendheid van DNA vaccins bij biggen te verhogen. Het laatste deel van dit hoofdstuk beschrijft het potentieel van DNA vaccins om een mucosale immuunrespons te induceren.

In de hoofdstukken 2 tot 5 wordt het experimentele werk van dit onderzoek weergegeven. Deze thesis had tot doel de volgende vragen te beantwoorden.

- Wat is de optimale route voor parenterale FaeG DNA vaccinatie, de intradermale (ID) of de intramusculaire (IM) route?
- Kan voorbehandeling met plasmide gecodeerd GM-CSF de immunogeniciteit van ons parenteraal DNA vaccin verhogen en is dit geassocieerd met een chemo-tactisch effect op APC?
- Kan de immunogeniciteit van het parenteraal FaeG DNA vaccin verder verhoogd worden door optimalisatie van de FaeG expressie?
- Kan parenterale priming met het geoptimaliseerd FaeG DNA vaccin in de aan- of afwezigheid van vitD₃ of plasmides coderend voor de A en B subeenheden van LT (samen de LT vectoren genoemd), op zichzelf of in combinatie met een orale F4 proteïne boost, de intestinale mucosale IgA respons induceren die nodig is om biggen te beschermen tegen PWD?

In hoofdstuk 2 werd de capaciteit van IM en ID injectie van pWRGFaeG om systemische cellulaire en humorale immuunresponsen te induceren bij gespeende biggen vergeleken met

als doel de optimale toedieningsweg te bepalen voor parenterale FaeG DNA vaccinatie. Eveneens werd het aduvans effect nagegaan van plasmide DNA coderend voor het porcine GM-CSF op ID DNA vaccinatie door dit 7 dagen voor de eerste immunisatie toe te dienen, aangezien aangetoond is dat plasmide gecodeerd GM-CSF APC aantrekt naar de injectieplaats bij muizen, met een maximaal effect na 7 dagen. De IM route induceerde betere humorale responsen aangezien IgG titers vlugger stegen en een zwakke IgA respons “geprimed” werd, terwijl de ID route superieur was in het induceren van celgemedieerde immuniteit, zoals aangetoond door een lymfocytenproliferatie test. ID toediening van het GM-CSF gen 7 dagen voor de eerste ID DNA vaccinatie verbeterde zowel de F4-specifieke cellulaire als humorale immuunresponsen en resulteerde zelfs in een efficiëntere priming van de IgA respons dan de IM immunisatie.

Daarom werd het aduvans effect van plasmide DNA coderend voor het porcine GM-CSF verder geëvalueerd op APC bij biggen (hoofdstuk 3). Er werd aangetoond dat ID toediening van het GM-CSF gen 7 dagen voor ID FaeG DNA vaccinatie het aantal epidermale CD1⁺ cellen (Langerhans’ cellen, DC van de huid) significant vermeerdeert ter hoogte van de immunisatieplaats. Dit was vergezeld van een verhoogd percentage APC ter hoogte van de immunologische inductieplaats (drainerende lymfeknoop) tot 4 dagen na de FaeG DNA vaccinatie. Deze resultaten suggereren niet alleen dat directe transfectie van APC en/of opname van het geëxprimeerde antigeen door deze cellen verhoogd zou kunnen zijn, maar ook dat de immuunrespons sneller kan geïnitieerd worden als GM-CSF DNA toegediend wordt voor de DNA vaccinatie. Bovendien, aangezien verondersteld wordt dat priming van een mucosale respons na parenterale immunisatie het gevolg is van migratie van antigeen-getransfecteerde APC naar mucosale inductieplaatsen, zal een stijgend aantal APC ook de kans op succesvolle mucosale priming verhogen. Daarom werd gekozen voor ID DNA vaccinatie voorafgegaan door GM-CSF injectie in de volgende studies.

In hoofdstuk 4 werd nagegaan of optimalisatie van de FaeG expressie en/of inclusie van de LT vectoren de immunogeniciteit van het FaeG DNA vaccin verder kan verhogen. Eveneens werd priming van een F4-specifieke intestinale mucosale immuunrespons nagegaan in een “ID DNA prime (2x) - orale F4 boost” protocol. Om de FaeG expressie te optimaliseren werd eerst een “Kozak” consensussequentie voor eukaryote translatie-initiatie ingevoegd in pcDNA1/*faeG19* (pcDNA1/*faeGK*). Vervolgens werd de pcDNA1 vector vervangen door pWRG7079 (pWRGFaeG). Deze vector bevat de “cytomegalovirus immediate early”

promotor, zoals pcDNA1, maar ook het geassocieerde intron A, dat werkt als een enhancer voor deze promotor en, in plaats van de “simian virus 40” polyadenylatie site (polyA) van pcDNA1, de gunstigere “bovine growth hormone” polyA voor terminatie van transcriptie. Bovendien bevat pWRG7079 ook de humane “tissue plasminogen activator” signaal sequentie voor extracellulaire secretie van het gecodeerde antigeen. Tenslotte werd het codon-gebruik van het bacteriële *faeG* gen aangepast aan dat van sterk geëxprimeerde genen bij het varken (pWRGFaeGopt). Zoals verwacht steeg de *in vitro* FaeG expressie in de orde pcDNA1/*faeG19*, pcDNA1/*faeGK*, pWRGFaeG, pWRGFaeGopt. Vervolgens werd de immunogeniciteit van pcDNA1/*faeG19*, pWRGFaeG en pWRGFaeGopt getest bij gespeende biggen in een “ID DNA prime – orale F4 boost” protocol. Na ID DNA vaccinatie werd een F4-specifieke serum IgG respons waargenomen waarvan niet alleen de sterkte maar ook de snelheid van aanzet verbeterd was in lijn met de stijgende FaeG expressie. Bijgevolg kon een F4-specifieke IgG respons zelfs al waargenomen worden vanaf de tweede week na de eerste immunisatie met pWRGFaeGopt. Bovendien induceerde enkel dit vector-codon-geoptimaliseerde construct een serum IgA respons. Toediening van de LT vectoren versterkte de antistoffenrespons nog verder en was noodzakelijk om een F4-specifieke cellulaire respons te induceren, maar deed de IgA respons teniet. Deze vectoren werden toegediend omdat LT gekend is voor het richten van een systemische respons naar een mucosale IgA respons bij muizen. Hier resulteerde de “ID DNA prime – orale F4 boost” echter voornamelijk in een systemische IgG respons. Slechts enkele biggen vertoonden een intestinale mucosale immuunrespons met antistoffen die hoofdzakelijk IgG waren in plaats van IgA. Normaal gezien biedt sIgA de voornaamste bescherming tegen intestinale pathogenen zoals ETEC. Desondanks resulteerde priming met pWRGFaeGopt in een significant gereduceerde faecale F4⁺ *E. coli* excretie terwijl toediening van de LT vectoren eveneens de duur van de faecale excretie verminderde. Deze data suggereren een rol voor IgG in deze partiële protectie.

In hoofdstuk 5 werd nagegaan of toediening van vitD₃ aan het geoptimaliseerde DNA vaccin de mucosale priming verder kon versterken en/of de antistoffenrespons kon richten naar IgA in ons “ID DNA prime (2x) – orale F4 proteïne boost” model. Om het belang van de orale F4 boost na te gaan in de inductie van een mucosale respons werd het heterologe “ID DNA prime (2x) – orale F4 proteïne boost” protocol vergeleken met een homologe ID DNA vaccinatie (3x) protocol. Een systemische IgG respons werd geïnduceerd door beide protocols, maar sterker door het heteroloog protocol. Toediening van de LT vectoren en, in mindere mate, van vitD₃ versterkte deze respons. Hoewel van LT en vitD₃ aangetoond is dat

ze een mucosale IgA respons induceren als ze toegediend worden aan parenterale proteïne vaccins konden we geen intestinale mucosale respons aantonen na parenterale DNA vaccinatie, onafhankelijk van de LT vectoren of vitD₃. Bovendien versterkte vitD₃ de systemische IgA respons geïnduceerd door pWRGFaeGopt niet. Na de orale F4 boost kon een zwakke mucosale IgG respons aangetoond worden. Deze mucosale respons varieerde aanzienlijk tussen de biggen, terwijl lage aantallen IgG antistoffen-secreterende cellen (ASC) meer algemeen konden gedetecteerd worden in de mesenteriale lymfeknopen van de DNA “geprimeerde” biggen, vooral als de LT vectoren of vitD₃ toegevoegd waren. Ondanks 2 DNA vaccinaties in aanwezigheid van deze adjuvantia en een orale F4 boost slaagden we er echter niet in om de sIgA respons te induceren die nodig is om bescherming te bieden tegen enterotoxigenic *E. coli*. In een tweede experiment werd de inductie van cytokine mRNA expressie en lymfocytenproliferatie geëvalueerd na ID DNA vaccinatie in aan- of afwezigheid van de LT vectoren of vitD₃. Een duidelijke FaeG-specifieke lymfocytenproliferatie kon enkel duidelijk aangetoond worden in aanwezigheid van de LT vectoren. Bij het aantonen van de cytokine mRNA expressie in de lokaal drainerende lymfeknopen werd een stijging in IL-6 en TGF- β mRNA expressie waargenomen na pWRGFaeGopt vaccinatie. Zowel de LT vectoren als vitD₃ versterkten de TGF- β mRNA expressie verder, terwijl de LT vectoren eveneens de IL-6 mRNA expressie leken te onderdrukken. Hoewel de IL-10 mRNA expressie sterk varieerde tussen de biggen werden verhoogde IL-10 mRNA niveaus steeds vergezeld door een onderdrukte FaeG-specifieke lymfocytenproliferatie. Dit is in overeenstemming met studies die een onderdrukte T-helper (Th)1- en Th2-cel proliferatie aantonen door IL-10 bij de mens en bij runderen. Bovendien zou de afwezigheid van een duidelijke IL-10 respons in de LT groep kunnen bijdragen aan de sterke FaeG-specifieke proliferatie die waargenomen werd in die groep. Aangezien IL-10 betrokken is in IgA isotype switching en IL-6 de IgA secretie bevordert zou de suppressie van deze cytokines door de LT vectoren ook de onderdrukte IgA respons kunnen verklaren die gezien werd in hoofdstuk 4.

Het laatste hoofdstuk (hoofdstuk 6) bevat de algemene discussie, de voornaamste conclusies en de toekomst perspectieven. In deze thesis werd aangetoond dat de immunogeniciteit van het parenteral FaeG DNA vaccin bij biggen sterk verbeterd kon worden door de optimale toedieningsroute te bepalen, de immunisatieplaats voor te behandelen met GM-CSF DNA en de FaeG expressie te optimaliseren. Niettemin resulteerde parenterale immunisatie met dit vaccin hoofdzakelijk in een systemische IgG respons en slaagde er zelfs in aanwezigheid van immunomodulators en/of een orale proteïne boost niet in om een

mucosale IgA respons te induceren of bescherming te bieden tegen infectie met F4⁺ *E. coli*. Om de sIgA respons te induceren die nodig is om bescherming te bieden tegen PWD via DNA vaccinatie zou de orale route mogelijks effectiever kunnen zijn. Hoewel orale DNA vaccinatie nog in zijn kinderschoenen staat zijn het gebruik van levende bacteriële dragers om het DNA vaccin af te leveren aan het darmgeassocieerde lymfoïd weefsel en formulaties die het DNA vaccin beschermen tegen degradatie en het richten naar de mucosale immunologische inductieplaatsen reeds getest bij muizen. Voornamelijk strategieën die het DNA vaccin selectief richten naar M cellen zouden veelbelovend kunnen zijn.

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CURRICULUM VITAE

Vesna Melkebeek werd geboren op 3 februari 1979 te Wetteren. In 1997 beëindigde zij haar secundaire opleiding, richting Wiskunde-Wetenschappen, aan het Sint-Gertrudis humaniora te Wetteren. In 1998 begon ze aan de studies biologie aan de Universiteit van Gent, waar ze twee jaar later het diploma behaalde van kandidaat in de biologie met onderscheiding. In 2002 studeerde ze met onderscheiding af als licentiaat in de biotechnologie. Vrijwel onmiddellijk daarna trad ze in dienst als wetenschappelijk medewerker aan het Laboratorium voor Immunologie van de Huisdieren, waar ze zich gedurende vier jaar verdiepte in de ontwikkeling van een DNA vaccin tegen F4⁺ enterotoxigene *Escherichia coli* infecties bij biggen. Dit onderzoek werd uitgevoerd onder leiding van Prof. Dr. E. Cox en Prof. Dr. B.M. Goddeeris en leidde tot dit proefschrift. Vesna is auteur of mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en nam actief deel aan verschillende nationale en internationale congressen.

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Verfaillie T, Melkebeek V, Snoek V, Douterlungne S, Cox E, Verdonck F, Vanrompay D, Goddeeris B, Cox E. (2004) Priming of piglets against enterotoxigenic *E. coli* F4 fimbriae by immunisation with FAEG DNA. *Vaccine*. 22:1640-1647.

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Melkebeek V., Van den Broeck W., Verdonck F., Goddeeris B.M., Cox E. (2007) Effect of plasmid DNA encoding the porcine granulocyte-macrophage colony-stimulating factor on antigen-presenting cells in pigs. *Vet Immunol Immunopathol*. Accepted with moderate revision.

ABSTRACTS AND POSTERS

Melkebeek V, Stuyven E, Cox E, Goddeeris BM. (2004) Intradermal injection of pigs with plasmid DNA encoding GM-CSF attracts antigen-presenting cells to porcine epidermis and improves the FaeG-specific lymphocyte proliferation to an FaeG DNA vaccine. Abstract and poster. 8th International Symposium on Dendritic Cells, October 17-21, Brugge, Belgium.

Melkebeek V, Cox E, Stuyven E, Goddeeris BM. (2004) Influence of delivery route and administration of plasmid DNA encoding the porcine GM-CSF on priming of piglets against F4+ enterotoxigenic *E. coli* following immunisation with FaeG DNA. Abstract and poster. DNA vaccines 2004 - The Gene Vaccine Conference, November 17-19, Monte Carlo, Monaco.

Cox E, Melkebeek V, Sonck E, Goddeeris B. Optimisation of an FaeG DNA vaccine to prime an F4-specific immune response in piglets. Abstract and poster. DNA vaccines 2004 - The Gene Vaccine Conference, November 17-19, Monte Carlo, Monaco.

Melkebeek V, Stuyven E, Goddeeris BM, Cox E. (2005) Intradermal delivery of plasmid DNA encoding the porcine GM-CSF recruits antigen-presenting cells to the injection site and enhances the immune response to an FaeG DNA vaccine. Abstract and poster. BSCDB Springmeeting on cell migration in cancer and immunity, May 27-28, Brussels, Belgium.

Melkebeek V, Sonck E, Goddeeris BM, Cox E. (2007) Optimization of the FaeG expression and the use of adjuvantia enhance priming by an FaeG DNA vaccine in pigs. Abstract and oral presentation. DNA vaccines 2007, May 23-25, Malaga, Spain.

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